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AUTHOR(S):

Ohshima, Toshihisa

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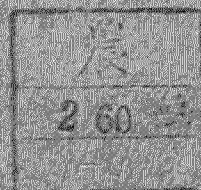
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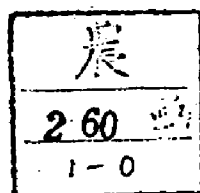


STUDIES ON BACTERIAL  
LEUCINE DEHYDROGENASE

TOSHIHISA OHSHIMA

1978

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## INTRODUCTION

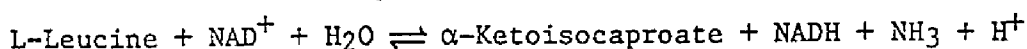
The enzymatic deamination of amino acids to the corresponding keto acids, which was first suggested by Neubauer (1) and Knoop (2), is of considerable significance in the metabolism of amino acids and includes two types of mechanism: transamination by transaminases and oxidative deamination by amino acid oxidases and amino acid dehydrogenases.

Amino acid dehydrogenases provide a route for incorporation of ammonia into organic compounds, i.e., a link between carbohydrate and amino acid metabolisms. Glutamate dehydrogenases (EC 1.4.1.2-4), which catalyze the interconversion of L-glutamate and  $\alpha$ -ketoglutarate, are recognized to be important because of the pivotal position in amino acids and organic acids metabolisms. The ubiquitous distribution of the enzymes reflects their metabolic significance. There are at least three types of the enzymes which differ in coenzyme specificity: those specific for either  $\text{NAD}^+$  (EC 1.4.1.2) or  $\text{NADP}^+$  (EC 1.4.1.4) and those that can function with both (EC 1.4.1.3) (3-4). These enzymes vary not only in coenzyme specificity, but also in some other properties, e.g., induction and repression of their syntheses by metabolites, regulation of activity by purine nucleotides and other ligands, and molecular properties (3-7). Thus, intensive studies on the molecular properties, primary structures and kinetic and regulatory mechanism of the enzymes have been carried out.

Almost all species of *Bacillus* are devoid of glutamate dehydrogenase but have alanine dehydrogenase (EC 1.4.1.1) and leucine dehydrogenase (EC 1.4.1.9) (8-11). Alanine dehydrogenase was first discovered in *B. subtilis* (12-13) and the physiological and enzymological properties were investigated by Yoshida and Freese (14). The physiological function of the enzyme in the carbon and nitrogen metabolism of bacilli also was shown. The enzyme was

found to participate in the spore germination of bacilli, which is triggered by L-alanine (15-16), and in the energy generation by the tricarboxylic acid cycle (17).

Leucine dehydrogenase (L-leucine: NAD<sup>+</sup> oxidoreductase, deaminating, EC 1.4.1.9) catalyzes the reversible deamination of L-leucine to  $\alpha$ -ketoisocaproate:



The dehydrogenase was first discovered in *B. cereus* (18) and partially purified from *B. subtilis* to investigate some of its properties (9). Hermier et al. (19) studied the enzyme from sporulating cells of *B. subtilis* and suggested that leucine dehydrogenase plays an important role as well as alanine dehydrogenase in spore germination (15). Obermeier and Poralla (20) reported recently that the enzyme functions catabolically in the metabolism of *B. subtilis* as described in Fig. 1.

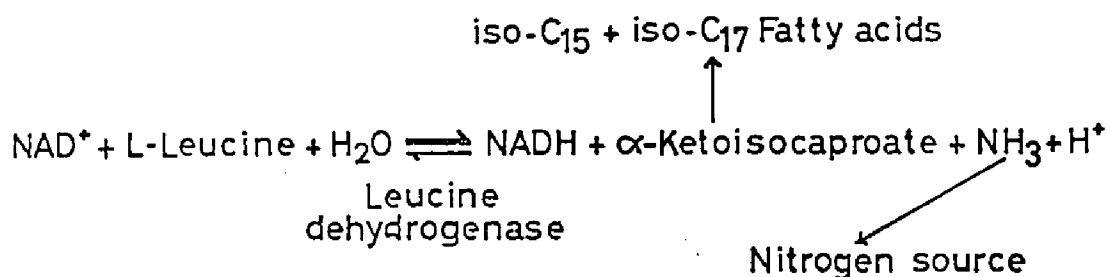


Fig. 1. The possible role of leucine dehydrogenase in *B. subtilis* for the production of precursors of iso-C<sub>15</sub> and iso-C<sub>17</sub> fatty acids and NH<sub>3</sub> proposed by Obermeier and Poralla.

Little information, however, is available about the molecular and catalytic properties of leucine dehydrogenase. In this thesis, the bacterial distribution, the purification and crystallization, enzymological and physicochemical properties, antitumor activity and some applications of leucine dehydrogenase are described.

## CHAPTER I

### DISTRIBUTION, PURIFICATION AND CRYSTALLIZATION OF LEUCINE DEHYDROGENASE

Leucine dehydrogenase was found in the vegetative cells and spores of *Bacillus* species (9, 15, 18). The enzyme was partially purified from *B. cereus* (18) and *B. subtilis* (9, 19) to show some enzymological properties.

In this chapter, the bacterial distribution of leucine dehydrogenase, and purification and crystallization of the enzyme from *B. sphaericus* are described.

### EXPERIMENTAL PROCEDURES

**Materials**  $\text{NAD}^+$ ,  $\text{NADH}$  and  $\text{NADP}^+$  were obtained from Kyowa Hakko Kogyo, Tokyo and amino acids were from Ajinomoto, Tokyo. DEAE-cellulose and Sephadex G-150 were purchased from Serva, Heidelberg and Pharmacia Fine Chemicals Uppsala, respectively. Hydroxyapatite was prepared according to the method of Tiselius et al. (21). The other chemicals were analytical grade reagents. 0.2 M Glycine-KCl-KOH buffer was made up by adding 2 M KOH to a mixture containing 0.2 M glycine and 0.2 M KCl to adjust the pH (22).

**Conditions for Cell Growth** The basal medium contained 1.5% peptone, 0.1% glycerol, 0.2%  $\text{KH}_2\text{PO}_4$ , 0.2%  $\text{K}_2\text{HPO}_4$ , 0.5% NaCl, 0.01% yeast extract, 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.01% meat extract in tap water. The pH was adjusted to about 7.2 with NaOH. The cultures for the studies on the distribution of leucine dehydrogenase were carried out with 50 ml-medium placed in a 500 ml flask on a reciprocating shaker at 27° for 15-36 h and a large scale culture was in a 30 liter jar fermentor at 30° for 20 h under aeration. The harvested cells were washed twice with 0.85% NaCl and subsequently with 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol. The washed cells were stored



frozen at  $-20^{\circ}$  until use.

*Enzyme Assay* The standard reaction mixture for the oxidative deamination contained 10  $\mu\text{mol}$  of L-leucine, 2.5  $\mu\text{mol}$  of  $\text{NAD}^{+}$ , 120  $\mu\text{mol}$  of glycine-KCl-KOH buffer (pH 10.7) and enzyme in a final volume of 0.8 ml. Enzyme was replaced by water in a blank. Incubation was carried out at  $25^{\circ}$  in a cuvette of a 1-cm light path. The reaction was started by addition of substrate (or enzyme) and followed by measuring the initial change in the absorbance at 340 nm with a Shimadzu MPS-50 L recording spectrophotometer. A quantity of the enzyme was used that would provide a linear change in the absorbance for at least 2 min.

*Definition of Unit and Protein Determination* One unit of the enzyme is defined as the amount of enzyme that catalyzes appearance of 1  $\mu\text{mol}$  of NADH, using a molar absorption coefficient of  $6,220 \text{ M}^{-1}\text{cm}^{-1}$  (23). Specific activity is expressed as units per mg of protein. Protein concentration of the enzyme was determined by the method of Lowry et al. (24) using egg albumin as a standard and with most column fractions, protein elution patterns were estimated by 280 nm absorption. Concentration of the crystalline enzyme was derived from the absorbance at 280 nm with a value of  $A_{1\text{ cm}}^{1\%} = 9.18$  obtained by absorbance and dry weight determinations. Spectrophotometer determination was made with a Carl Zeiss PMQ II spectrophotometer with a 1-cm light path.

*Ultracentrifugal Analysis* The purity of the enzyme and its sedimentation coefficient were examined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm (25).

*Disc Gel Electrophoresis* Disc gel electrophoresis was performed by the procedure of Davis (26). The gels were stained with 1% Amino-black in 7% acetic acid and destained electrophoretically in 7% acetic acid.

## RESULTS

*Bacterial Distribution of Leucine Dehydrogenase* Screening was carried out in order to find out bacterial strains that would produce a high activity of leucine dehydrogenase. As shown in Table I, the high enzyme activity occurs mainly in *Bacillus* species, especially in *Bacillus sphaericus*, *B. cereus*, *B. megaterium* and *B. subtilis*. The slight activity was found in *Corynebacterium sepedonicum*, *C. pseudodiphtheriticum* and *Alcaligenes faecalis*. *B. sphaericus* (IFO 3525) in which occurs leucine dehydrogenase most abundantly was chosen for the purpose of purification of the enzyme. The extract of *B. sphaericus* (IFO 3525) possessed a potent alanine dehydrogenase (specific activity; 0.41), but no  $\text{NAD}^+$  and  $\text{NADP}^+$ -specific glutamate dehydrogenases.

Table I. *Distribution of leucine dehydrogenase in various strains of bacteria*

Strains	Specific activity
<i>Bacillus sphaericus</i> (IFO 3525)	0.42
<i>sphaericus</i> (IFO 3526)	0.31
<i>cereus</i> (AKU 1311)	0.40
<i>megaterium</i> (AKU 1341)	0.40
<i>subtilis</i> var. <i>niger</i> (IFO 3108)	0.30
<i>subtilis</i> (IFO 3037)	0.10
<i>subtilis</i> var. <i>aterrimus</i> (IFO 3214)	0.05
<i>subtilis</i> (IFO 3009)	0.14
<i>brevis</i> (IFO 3331)	0.18
<i>mesentericus</i> var. <i>flavus</i> (IFO 3028)	0.12
<i>roseus</i> (IFO 3041)	0.09
<i>aneurinolyticus</i> (ICR 1320)	0.04

(Table I continued)

Strains	Specific activity
<i>Bacillus licheniformis</i> (IFO 12200)	0.21
<i>Corynebacterium sepedonicum</i> (IFO 3306)	0.06
<i>pseudodiphtheriticum</i> (ICR 2210)	0.06
<i>Alcaligenes faecalis</i> (IAM B-141-1)	0.03

No activity was found in the following organisms: *Escherichia coli* (ICR 0010), *Aerobacter aerogenes* (IFO 3320), *Erwinia aroideae* (IFO 3830), *Serratia marcescens* (IFO 3046), *Proteus vulgaris* (IFO 3045), *Proteus mirabilis* (IFO 3849), *Agrobacterium radiobacter* (IAM 1526), *Agrobacterium tumefaciens* (IAM B-26-1), *Flavobacterium lutescens* (IFO 3084), *Micrococcus luteus* (ICR 1850), *Micrococcus roseus* (IFO 3764), *Sarcina lutea* (IAM 1099), *Flavobacterium lutescens* (IFO 3085), *Flavobacterium gasogenes* (IFO 12065), *Bacterium mycoides* (IFO 3040), *Pseudomonas riboflavina* (IFO 3140), *Pseudomonas aeruginosa* (IFO 3924) and *Pseudomonas schuylkilliensis* (IFO 12055).

*Purification of Leucine Dehydrogenase* All operations were performed at 0-5° and 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol was used in the purification procedures, unless otherwise stated.

*Step 1. Preparation of Crude Extract* The washed cells (about 2 kg; wet weight) were suspended in 2 liter of the buffer and subjected in 300 ml portions to sonication for 30 min in a 19 kHz oscillator followed by centrifugation and dialysis against the buffer.

*Step 2. Protamine Sulfate Treatment* To the cell-free extract

was added 1.0 ml of 2% protamine sulfate solution (pH 7.2) per 100 mg of the protein with stirring. After about 12 h, the precipitate was removed by centrifugation.

Step 3. *Ammonium Sulfate Fractionation* The supernatant solution (8,970 ml) was brought to 30% saturation with ammonium sulfate, and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant solution to 70% saturation. The pH was kept at about pH 7.2 with 10%  $\text{NH}_4\text{OH}$ . The precipitate obtained by centrifugation was dissolved in the minimum volume of the buffer. The enzyme solution was dialyzed against 100 volumes of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step 4. *DEAE-Cellulose Column Chromatography* The enzyme solution (875 ml) was applied to a DEAE-cellulose column (10 X 60 cm) equilibrated with the buffer. After the column was washed with the buffer and then with the buffer containing 0.23 M NaCl, the enzyme was eluted with the buffer supplemented with 0.40 M NaCl. The flow rate was 150 ml per h and 30-ml fractions were collected. The active fractions were pooled, concentrated by addition of ammonium sulfate (70% saturation), and dialyzed against two changes of 100 volumes of 0.001 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol.

Step 5. *Hydroxyapatite Column Chromatography* The enzyme solution (260 ml) was applied to a hydroxyapatite column (4 X 40 cm) equilibrated with 0.001 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol. After column was washed with the same buffer, the enzyme was eluted with 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol. The active fractions were collected and concentrated by ammonium sulfate (70% saturation). The precipitate was dissolved in a small volume of the buffer.

Step 6. *Sephadex G-150 Column Chromatography* The enzyme solution (89.5 ml) was applied in about 7-ml portions to Sephadex G-150 column (2.5 X 100 cm) equilibrated with the buffer, and eluted with the same buffer. The active fractions were pooled and concentrated by addition of ammonium sulfate (70% saturation). The precipitate was dissolved in a small volume of the buffer and Sephadex G-150 column chromatography (Fig. 2) and precipitation with ammonium sulfate were repeated as described above. After centrifugation, the precipitate was dissolved in a minimum volume of the buffer.

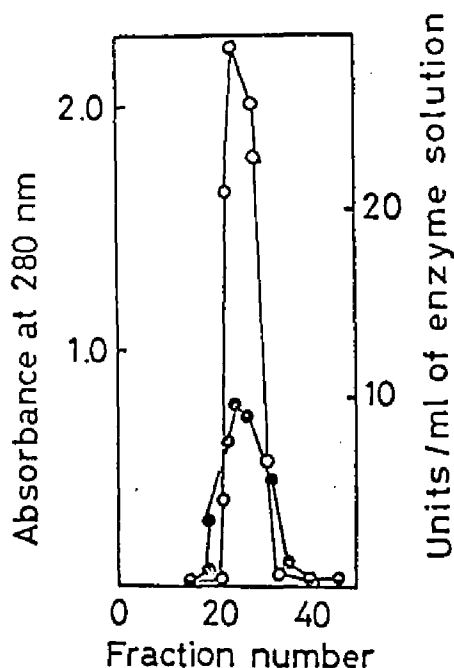


Fig. 2. The elution pattern from a Sephadex G-150 column. The flow rate was about 10 ml per h and 5 ml fractions were collected. (●): Absorbance at 280 nm, (○): Units/ml of the enzyme.

Step 7. *Crystallization* Ammonium sulfate was added slowly to the enzyme solution until a faint turbidity was obtained. The

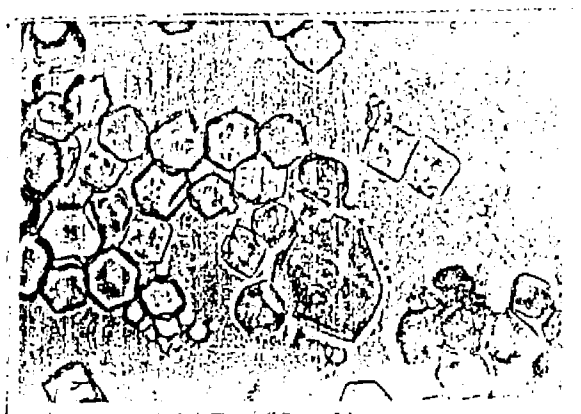


Fig. 3. Crystals of leucine dehydrogenase

Table II. *Purification of leucine dehydrogenase*

Steps	Total Protein	Specific activity	Total units	Yield
	mg	units/mg		%
Crude extract	161,000	0.21	33,980	100
Protamine treatment	90,600	0.35	31,710	94
Ammonium sulfate fractionation	47,200	0.54	25,490	75
DEAE-cellulose	7,250	2.39	17,330	51
Hydroxyapatite	2,740	5.59	15,320	45
Sephadex G-150 (1)	1,020	14.5	14,740	43
Sephadex G-150 (2)	469	26.5	12,430	37
Crystallization	254	42.3	10,750	32

pH of the solution was kept constant at 7.2-7.5 with 10%  $\text{NH}_4\text{OH}$  solution. On standing at  $4^\circ$  overnight, crystal formation occurred. The crystals took the form of the rhombic dodecahedrons (Fig. 3).

Approximately 200-fold purification was achieved with an overall yield of 32 %. A protocol of the purification was presented in Table II.

The crystalline enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis

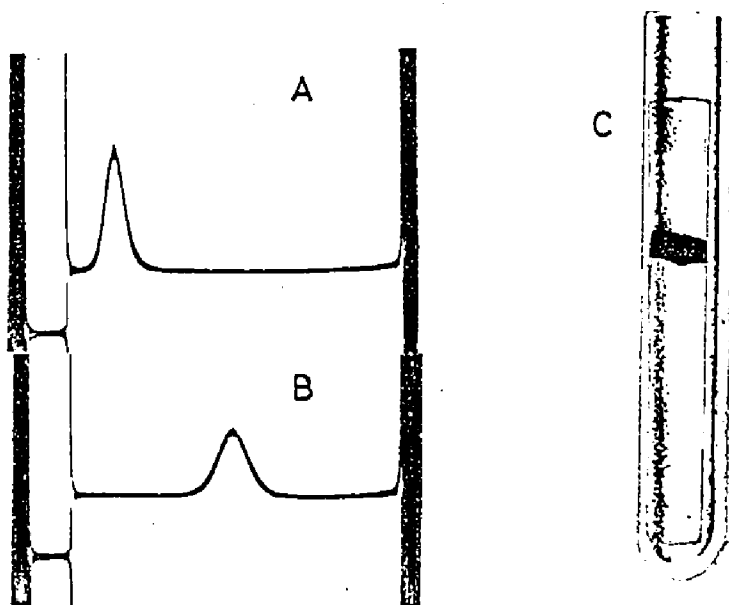


Fig. 4. Sedimentation patterns (A and B) and disc gel electrophoresis (C) of leucine dehydrogenase.

Sedimentation patterns were obtained at 6 mg/ml of protein concentration in 0.01 M potassium phosphate buffer (pH 7.2). Pictures were taken at 28 min (A) and 76 min (B) after achieving top speed (59,780 rpm).

(Fig. 4). The sedimentation coefficient of the enzyme, calculated for water at 20° and zero concentration, is 9.0 S.

#### DISCUSSION

Amino acid dehydrogenases play an important role in nitrogen metabolism. Glutamate dehydrogenases are widely distributed in organisms (3). Many species of *Bacillus*, however, are devoid of glutamate dehydrogenase and possess alanine dehydrogenase and leucine dehydrogenase (8-11). These dehydrogenases participate in spore germination (15-16). The results on the distribution of leucine dehydrogenase showed that the enzyme occurs almost exclusively in *Bacillus* species. Leucine dehydrogenase was purified to homogeneity and crystallized from *B. sphaericus* (IFO 3525) which has the highest activity to investigate the physicochemical and enzymological properties. The crystalline enzyme preparation was found to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis.

#### SUMMARY

Leucine dehydrogenase occurred mainly in *Bacillus* species, and most abundantly in *B. sphaericus* (IFO 3525). The enzyme was purified to homogeneity and crystallized from *B. sphaericus* (IFO 3525) with an over all yield of about 30%. The purification was carried out by the following steps; sonic extraction, protamine sulfate treatment, ammonium sulfate fractionation, DEAE-cellulose column chromatography, hydroxyapatite column chromatography and Sephadex G-150 column chromatography. The crystals of the enzyme took the form of the rhombic dodecahedrons. The enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. The sedimentation coefficient ( $S_{20, w}^0$ ) of the enzyme was determined to be 9.0 S.



## CHAPTER II

### PROPERTIES OF LEUCINE DEHYDROGENASE FROM *BACILLUS SPHAERICUS*

Preparation of crystalline leucine dehydrogenase from the cell-free extract of *B. sphaericus* was described in CHAPTER I.

In this chapter, the physicochemical and enzymological properties of the crystalline enzyme are described.

### EXPERIMENTAL PROCEDURES

**Materials**  $\text{NAD}^+$ ,  $\text{NADH}$ ,  $\text{NADP}^+$ , nucleosides, nucleotides and purine and pyrimidine bases were obtained from Kyowa Hakko Kogyo, Tokyo, amino acids from Ajinomoto, Tokyo, leucine analogues from Nutritional Biochemistry,  $\text{NAD}^+$ -analogues from P-L Biochemicals, Inc.,  $\alpha$ -keto acids (sodium salt) from Sigma Chemical and Sephadex G-25 from Pharmacia, Uppsala, respectively. An  $\text{NADP}^+$ -analogue (27), which is the compound phosphorylated at 2' or 3' position of the nicotinamide ribose of  $\text{NAD}^+$ , was the generous gift of Dr. M. Kuwahara, Department of Food Science, Kagawa University, Kagawa. *p*-Chloromercuribenzoate and urea were purified by crystallization from alkaline solution by acidification and from ethanol, respectively, before use. The other chemicals were analytical grade reagents.

**Enzyme Preparation** The enzyme was purified from a cell-free extract of *B. sphaericus* (IFO 3525) and crystallized as described in CHAPTER I.

**Assay of Leucine Dehydrogenase** The oxidative deamination was determined as described in CHAPTER I. The assay system for the reductive amination consisted of 10  $\mu\text{mol}$  of sodium  $\alpha$ -ketoisocaproate, 0.1  $\mu\text{mol}$  of  $\text{NADH}$ , 750  $\mu\text{mol}$  of  $\text{NH}_4\text{Cl}$ - $\text{NH}_4\text{OH}$  buffer (pH 9.5) and the enzyme in a volume of 1.0 ml. Incubation was carried out at 25° in a cuvette of a 1-cm light path. the activity was assayed by monitoring a initial decrease in the absorbance at 340 nm.

Assays with coenzyme analogues were conducted by measuring increase in the absorbance at the following wavelengths: 3-acetylpyridine-NAD<sup>+</sup>, 363 nm (a molar absorption coefficient,  $M^{-1}cm^{-1}$ ,  $\epsilon = 9.1 \times 10^3$ ); deamino NAD<sup>+</sup>, 338 nm ( $\epsilon = 6.2 \times 10^3$ ); 3-acetylpyridine-deamino NAD<sup>+</sup>, 361 nm ( $\epsilon = 9.0 \times 10^3$ ); 3-pyridinealdehyde-NAD<sup>+</sup>, 358 nm ( $\epsilon = 9.3 \times 10^3$ ); thionicotinamide-NAD<sup>+</sup>, 395 nm ( $\epsilon = 11.3 \times 10^3$ ) (28-29); and NADP<sup>+</sup>-analogue, 340 nm ( $\epsilon = 6.2 \times 10^3$ ) (27). A quantity of enzyme was used that would provide a linear change in the absorbance for at least 2 min.

*Definition of Unit and Protein Determination* Unit and specific activity were defined as above (CHAPTER I) and protein was determined by measuring the absorbance at 280 nm ( $A_{1\%}^{1cm} = 9.18$ ).

*Sedimentation Equilibrium Analysis* The molecular weight of the enzyme was determined by ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (25). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operation was employed in order to perform the experiment on five samples of different initial concentration ranging from 0.034 to 0.17% with the use of An-G rotor and double-sector cells of different sidewedge angles. The rotor was centrifuged at 6,166 or 4,600 rpm at 20°. Interference patterns were photographed at intervals of 30 min to compare and make sure that the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined using the synthetic cell.

*Disc Gel Electrophoresis in the Presence of Sodium Lauryl Sulfate* The samples of proteins were treated with 1% sodium lauryl sulfate and 1% 2-mercaptoethanol at 37° for 2 h. Disc gel electrophoresis in the presence of 0.1% sodium lauryl sulfate was carried out according to the method of Weber and Osborn (30).

#### *Determination of Amino-Terminal Residue by Dansylation*

Dansylation of the enzyme was carried out according to the method of Weiner *et al.* (31). Dansyl chloride (0.03 ml, 20 mg/ml) was added to the mixture of the enzyme (100  $\mu$ g), sodium lauryl sulfate (1 mg) and 25  $\mu$ mol of sodium bicarbonate (pH 9.8) in a volume of 0.9 ml. After incubation at 37° for 1 h, 0.2 ml of 20% trichloroacetic acid was added to the reaction mixture to precipitate the protein. The precipitate obtained by centrifugation was washed several times with 1 N HCl and hydrolyzed with 6 N HCl at 105° for 12 h. The hydrolysate was dried in a vacuum at 45°. The residue was dissolved in a small volume of 50% pyridine and subjected to thin layer chromatography on polyamide plate (5 X 5 cm). Two-dimensional chromatography was performed with the three solvent systems; Solvent 1: 1.5% formic acid in water, Solvent 2: benzene-acetic acid (9:1), and Solvent 3: ethyl acetate-acetic acid-methanol (20:1:1). Dansylated amino acids were visualized under the ultraviolet light and identified by comparison with the standard dansyl amino acids.

*Determination of Carboxyl-Terminal Residue* Carboxyl-terminal amino acids were determined by tritium labelling method of Matsuo *et al.* (32). The enzyme (about 1 mg) was dissolved in 0.1 ml of tritiated water (50 mCi) and 0.2 ml of pyridine. Acetic anhydride (0.05 ml) was added slowly at 0° and pyridine (0.2 ml) and acetic anhydride (0.05 ml) were further added to the reaction mixture. After the reaction was incubated at room temperature for 20 h, the mixture was lyophilized. The residue was dissolved in a small volume of water and lyophilized again. These treatments were repeated five times to remove thoroughly tritiated water. The tritiated protein was hydrolyzed with 0.5 ml of 6 N HCl at 105° for 48 h. The hydrolysate was subjected to two-dimensional thin layer chromatography on a cellulose plate

(20 X 20 cm) with the solvent system described by Jones and Heathcote (33). The amino acids on the chromatogram were detected with 2,4-dinitrofluorobenzene (34). The spots of amino acids were cut off and transferred to scintillation vials containing the scintillation fluid of toluene system. The radioactivity was measured with a Tri-Carb Liquid Scintillation Counter 3320.

*Determination of Free Sulfhydryl Groups of Leucine Dehydrogenase* The enzyme solution was exhaustively dialyzed against 0.01 M potassium phosphate buffer (pH 7.2) which was free from 2-mercaptoethanol. The reaction of the enzyme with a sulfhydryl reagent was carried out at 25° in a cuvette of 1-cm light path or in a small test tube, and the enzyme activity of the mixture was measured with a reaction system containing L-leucine under the standard conditions.

Titration of reactive sulfhydryl groups with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was performed by the method of Ellman (35). The protein (0.3-0.6 mg) was dissolved in 0.1 M potassium phosphate buffer (pH 8.0) in a final volume of 0.6 ml. The reaction was started by addition of a freshly prepared DTNB solution in about 50-fold excess. The absorbance of the mixture was followed at 412 nm until no further increase in the absorbance was observed. The number of sulfhydryl groups was calculated from the absorbance at 412 nm using the molar absorption coefficient of  $13,600 \text{ M}^{-1}\text{cm}^{-1}$  (35).

Free sulfhydryl groups were also determined with 4,4'-dithiodipyridine (4-PDS) by measuring an increase in the absorbance at 324 nm (36). The number of sulfhydryl groups was calculated on the basis of the molar absorption coefficient of  $19,800 \text{ M}^{-1}\text{cm}^{-1}$ .

The determination of sulfhydryl groups with p-chloromercuribenzoate (pCMB) was carried out by measuring an increase in the

absorbance at 250 nm (the molar absorption coefficient of 7,600  $M^{-1}cm^{-1}$ ) according to the method of Boyer (37).

The reactions of  $HgCl_2$  and N-ethylmaleimide with the sulfhydryl groups of enzyme were followed by measuring the enzyme activity.

*Gel Filtration Studies* The binding of NADH to leucine dehydrogenase was studied at 25° by the gel filtration technique of Hummel and Dreyer (38). Samples (0.8 ml) containing enzyme and NADH were applied to a Sephadex G-25 column (0.9 X 57 cm). The column was equilibrated with 0.1 M potassium phosphate buffer (pH 8.0) containing the same concentration of NADH. The elution was performed with the same buffer. The eluate was collected at the rate of about 1.5 ml per min in 1.2-1.6 ml fraction. The concentration of NADH in the eluate was determined spectrophotometrically at 340 nm, with a Carl Zeiss PMQ II spectrophotometer with a 1.0 cm-light path, by using a molar absorption coefficient of 6,220  $M^{-1}cm^{-1}$  (23), which was applied to free NADH and NADH bound to the enzyme.

*Fluorescence Measurements* Fluorescence measurements were conducted with a Shimadzu Corrected Recording Spectrofluorophotometer RF-502 using Quartz cells filled with 2.5 ml-sample. The experimental data for titration of the binding sites of NADH to enzyme were analysed in a similar way (39-41). The number of binding sites (n) was calculated by the following equation and deduced from the graph of the Klotz equation (42).

$$\frac{1}{1-Y} = \frac{1}{K_d} \left( \frac{[NADH]}{Y} - n[E_t] \right), \quad Y = \frac{\Delta F}{\Delta F_{max}}$$

where Y = fraction of sites occupied for a total NADH concentration [NADH],  $\Delta F$  = actual fluorescence change for a given concentration of added NADH,  $\Delta F_{max}$  = maximum change in observed fluorescence, corresponding to complete saturation of the sites

with NADH, and  $K_d$  = dissociation constant of NADH.

## RESULTS

*Absorption Spectrum of the Enzyme* The absorption spectrum of leucine dehydrogenase showed an absorption maximum only at 280 nm. The absorption coefficient ( $A_{1\%}^{1\text{cm}}$ ) was obtained to be 9.18 by absorbance and dry weight determinations. The absorption ratio at 280 and 260 nm was calculated to be 1.62.

*Equilibrium Constant of Leucine Dehydrogenase Reaction* Equilibrium constant of the enzyme reaction,  $K_{eq}$  is defined as follows.

$$K_{eq} = \frac{[\text{NADH}][\alpha\text{-Ketoisocaproate}][\text{Ammonia}][\text{H}^+]}{[\text{NAD}^+][\text{L-Leucine}]}$$

The  $K_{eq}$  values were determined to be  $1.2 \times 10^{-14}$  and  $5.3 \times 10^{-16}$  at pH 9.5 and 10.7, respectively, at 25°.

*Stability of the Enzyme* The enzyme can be stored at 4° as a suspension in 70% saturated ammonium sulfate (pH 7.2) without loss of activity for periods over 3 years. The purified enzyme was stable up to 60° when heated for 5 min in 0.01 M potassium phosphate buffer (pH 7.2).

The enzyme was very stable at 6.5 to 9.0 when incubated at 50° for 5 min. A freezing and thawing of the enzyme solution, however, caused a slight decrease in the activity.

*Effect of Temperature and pH on the Enzyme Activity* When the oxidative deamination of L-leucine was investigated at various temperatures, the enzyme activity increased with increasing temperature up to 60°, and above 65° the activity was rapidly decreased.

The enzyme shows a maximum reactivity in the pH range of 10.5-10.8 for the oxidative deamination of L-leucine, L-valine and L-isoleucine, when examined in the presence of 0.15 M glycine

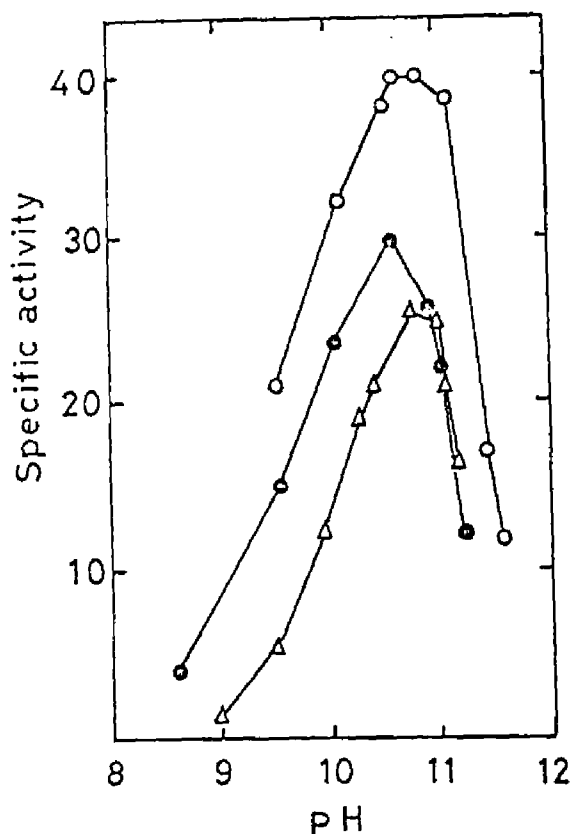


Fig. 5. Effect of pH on the oxidative deamination. Composition of reaction mixture was 2.5 mM  $\text{NAD}^+$  and 10 mM L-leucine (o-o), L-valine (●-●) or L-isoleucine ( $\Delta$ - $\Delta$ ) in 0.15 M glycine-KCl-KOH buffer.

KCl-KOH buffer (Fig. 5). The rates of the enzymatic deaminations decline markedly above pH 11 and below pH 10. The pH optima for the reductive amination of  $\alpha$ -ketoisovalerate and  $\alpha$ -ketovalerate, and  $\alpha$ -ketoisocaproate are 9.0 and 9.5, respectively, in the presence of 0.75 M  $\text{NH}_4\text{Cl}$ - $\text{NH}_4\text{OH}$  buffer (Fig. 6). The rates of the enzymatic amination reactions decline markedly above pH 10.

**Substrate Specificity** The ability of the enzyme to catalyze the oxidative deamination of various amino acids is presented in Table III. In addition to L-leucine, L-valine and L-isoleucine,

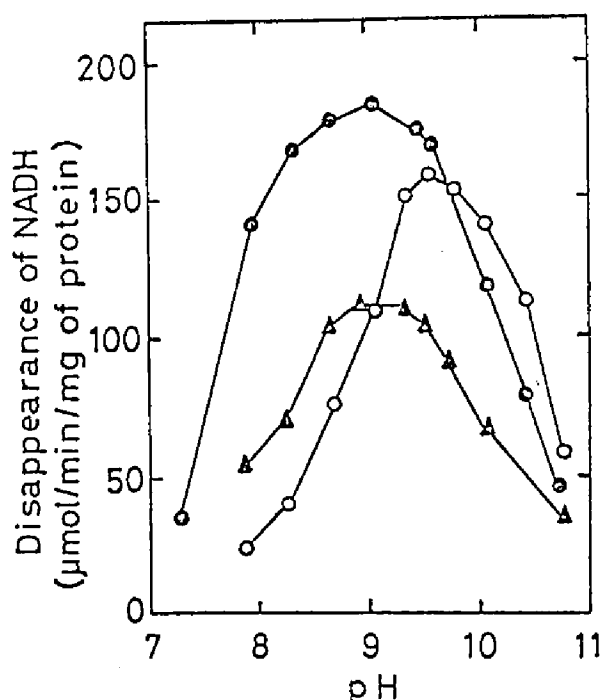


Fig 6. Effect of pH on the reductive amination. The reaction mixtures contained 0.1 mM NADH and 10 mM  $\alpha$ -Ketoisocaproate (o-o),  $\alpha$ -ketoisovalerate (●-●) or  $\alpha$ -ketovalerate (▲-▲) in 0.75 M  $\text{NH}_4\text{Cl}$ - $\text{NH}_4\text{OH}$  buffer.

which are the preferred substrates, straight-chain aliphatic L-amino acids such as L-norvaline and L- $\alpha$ -aminobutyrate also are effectively deaminated. D-Amino acids, L-alanine, L-glutamate, L-threonine, L-phenylalanine and amino acids other than  $\alpha$ -amino acid are not substrates.

The substrate specificity of the enzyme for the reductive amination is given in Table IV. All of the keto analogues of the substrates for the oxidative deamination serve as good substrates for the amination reaction. The reactivity of  $\alpha$ -ketoisovalerate, a keto analogue of valine, is higher than that of  $\alpha$ -ketoisocaproate, a keto analogue of leucine, which is the



Table III. Substrate specificity for the oxidative deamination.

Substrates <sup>a</sup> (10 mM)	Relative activity	K <sub>m</sub> (mM)
L-Leucine	100	1.0 <sup>b</sup>
L-Valine	74	1.7 <sup>b</sup>
L-Isoleucine	58	1.8 <sup>b</sup>
L-Norvaline	41	3.5 <sup>c</sup>
L- $\alpha$ -Aminobutyrate	14	10.0 <sup>c</sup>
L-Norleucine	10	6.3 <sup>c</sup>
$\gamma$ -Methylallylglycine	8.2	-
tert-DL-Leucine	1.6	-
S-Methyl-L-cysteine	1.4	-
L-4-Azaleucine	0.6	-
L-Methionine	0.6	-
L-Cysteine	0.3	-
L-Penicillamine	0.2	-
S-Ethyl-L-cysteine	0.1	-

<sup>a</sup> Inert: cycloleucine, L-cysteic acid, S-methyl-L-methionine, L-alanine, L-glutamate, L-threonine, L-serine, glycine, L-phenylalanine, L-ethionine, L-lysine, L-arginine, D-leucine, D-valine, D-alloisoleucine, D-isoleucine, D- $\alpha$ -aminobutyrate, D-norvaline, D-norleucine, glycylleucine,  $\epsilon$ -amino-n-caproate, 7-aminoheptanoate,  $\beta$ -alanine and  $\beta$ -aminovalerate.

<sup>b</sup> The K<sub>m</sub> value was obtained from the secondary plots of intercepts versus reciprocal concentrations of the substrate.

<sup>c</sup> The apparent K<sub>m</sub> value was determined by Lineweaver-Burk plots with reaction system containing 2.5 mM NAD<sup>+</sup>.

Table IV. *Substrate specificity for the reductive amination.*

Substrates <sup>a</sup> (10 mM)	Relative activity	K <sub>m</sub> (mM)
α-Ketoisocaproate	100	0.31 <sup>b</sup>
α-Ketoisovalerate	126	1.4 <sup>b</sup>
α-Ketovalerate	76	1.7 <sup>b</sup>
α-Ketobutyrate	57	7.7 <sup>c</sup>
α-Ketocaproate	46	7.0 <sup>c</sup>

<sup>a</sup> Inert: pyruvate, α-ketoglutarate, phenylpyruvate, oxalacetate and glyoxylate.

<sup>b</sup> The K<sub>m</sub> value was obtained from the secondary plots of intercepts versus reciprocal concentrations of the substrate.

<sup>c</sup> The apparent K<sub>m</sub> value was determined by Lineweaver-Burk plots with the reaction system containing 0.1 mM NADH and 0.75 M NH<sub>4</sub>Cl-NH<sub>4</sub>OH buffer (pH 9.5).

best substrate for the deamination reaction.

Ammonia is the exclusive substrate as an amino donor for the reductive amination of α-ketoisocaproate. None of 0.25 M Tris-HCl, hydroxylamine, methylamine and ethylamine, 0.15 M ethylenediamine and ε-aminocaproate, and 0.1 M L-glutamine, L-asparagine and D-glutamine were found to be substrates in the α-ketoisocaproate system under the standard conditions except that 250 μmol of glycine-KCl-KOH buffer (pH 9.5) was used.

*Coenzyme Specificity* The enzyme requires NAD<sup>+</sup> as a natural coenzyme for the oxidative deamination of L-leucine, and NADP<sup>+</sup> is inert (Table V). NADP<sup>+</sup> has no effect on the NAD<sup>+</sup>-dependent

Table V. Coenzyme specificity.

The reaction was carried out at pH 9.5 in order to avoid degradation of NAD<sup>+</sup>-analogues at strongly alkaline pH.

Coenzymes (2.5 mM)	Relative activity	K <sub>m</sub> (mM)
NAD <sup>+</sup>	100	0.39 <sup>a</sup>
NADP <sup>+</sup>	0	-
NAD <sup>+</sup> + NADP <sup>+</sup>	92	-
NADP <sup>+</sup> -analogue	0	-
3-Acetylpyridine-NAD <sup>+</sup>	166	0.77 <sup>b</sup>
Deamino NAD <sup>+</sup>	81	0.71 <sup>b</sup>
3-Acetylpyridine-deamino NAD <sup>+</sup>	100	2.41 <sup>b</sup>
3-Pyridinealdehyde-NAD <sup>+</sup>	19	-
Thionicotinamide-NAD <sup>+</sup>	21	-

<sup>a</sup> The K<sub>m</sub> value was obtained from the secondary plots of intercepts *versus* reciprocal concentrations of the substrate.

<sup>b</sup> The apparent K<sub>m</sub> value was determined by Lineweaver-Burk plots with the reaction system containing 10 mM L-leucine.

deamination of L-leucine. In addition, some analogues of NAD<sup>+</sup> serve as a coenzyme (Table V). 3-Acetylpyridine-NAD<sup>+</sup>, deamino NAD<sup>+</sup> and 3-acetylpyridine-deamino NAD<sup>+</sup> show the relatively high activity, but their affinities for the enzyme are lower than NAD<sup>+</sup>.

**Inhibitors** The effects of non-substrate amino acids and several other compounds on the oxidative deamination of L-leucine are given in Tables VI and VII. The enzyme dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.2) was used. D-Enantiomers of the substrate amino acids behave as competitive

Table VI. *Effect of non-substrate amino acids on the oxidative deamination.*

The enzyme was assayed with a reaction system containing L-leucine under the standard conditions after preincubated with the amino acids listed at pH 10.7 and 25° for 5 min.

Amino acid (10 mM)	Relative activity	Ki (mM)
None	100	-
D-Leucine	69	2.2
D-Valine	85	10.8
D-Alloisoleucine	89	7.9
D-Norleucine	91	52.6
D-Norvaline	88	3.0
D- $\alpha$ -Aminobutyrate	93	9.4
L-Alanine	97	-
L-Glutamate	99	-
L-Aspartate	97	-
L-Serine	100	-
L-Threonine	100	-
L-Phenylalanine	98	-
L-Lysine	99	-
L-Arginine	100	-
L-Proline	100	-
Glycine	100	-

inhibitors against L-leucine. The inhibition constants for D-leucine and D-norvaline are relatively low. L-Alanine, L-glutamate and other non-substrate L-amino acids are not inhibitory.

Table VII. *Effect of inhibitors on the oxidative deamination of L-leucine.*

After the enzyme was preincubated with the compounds at 25° for 10 min, the activity was determined with a reaction system containing L-leucine under the standard conditions.

Inhibitors	Concentration (mM)	Relative activity
None	-	100
pCMB	0.012	18
HgCl <sub>2</sub>	0.01	5
CuSO <sub>4</sub>	1	64
CoCl <sub>2</sub>	1	61
MgCl <sub>2</sub>	1	77
MnCl <sub>2</sub>	1	90
EDTA	1	100
$\alpha,\alpha'$ -Dipyridyl	1	100
NaN <sub>3</sub>	10	98
Sodium arsenate	10	100
Na <sub>2</sub> S	0.1	5

The enzyme was strongly inhibited by pCMB and HgCl<sub>2</sub>, typical inhibitors of sulfhydryl-enzymes. The inhibition was recovered partially by 2-mercaptoethanol, dithiothreitol and L-cysteine. Some metal ions such as Cu<sup>2+</sup>, Co<sup>2+</sup> and Mg<sup>2+</sup> were inhibitory, but not at all in the presence of 0.01% 2-mercaptoethanol. EDTA and  $\alpha,\alpha'$ -dipyridyl have no effect on the oxidative deamination of L-leucine.

When the enzyme was preincubated with the various compounds

(1 mM) at 25° and pH 10.7 for 5 min and assayed with the deamination system of L-leucine, none of the following purine and pyrimidine bases, nucleosides and nucleotides showed appreciable influence on the activity: adenine, adenosine, AMP, ADP, ATP, guanosine, GMP, GTP, cytosine, thymine, FAD and FMN.

The effect of vitamin B<sub>6</sub> compounds on the activity was also investigated. The enzyme was preincubated with 1 mM vitamin B<sub>6</sub> compounds in 50 mM potassium phosphate buffer (pH 7.2) at 37° for 1 h, and then assayed with the oxidative deamination system of L-leucine. The preincubation with pyridoxal 5'-phosphate led to about 40% loss of the activity, which was restored almost fully by exhaustive dialysis against two changes of 500 volumes of 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol for about 20 h. Pyridoxal, pyridoxamine 5'-phosphate, salicylaldehyde, glyoxylate and acetaldehyde were ineffective.

The addition of pyridoxal 5'-phosphate to the enzyme, which shows an absorption spectrum of simple protein, led to appearance of absorption peaks at 340 and 427 nm. The enzyme incubated with 1 mM pyridoxal 5'-phosphate was reduced with 5 mM sodium borohydride at 5° for 10 min according to Matsuo and Greenberg (43) and dialyzed against two changes of 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol for 20 h. The 427 nm peak disappeared and the absorbance around 340 nm increased with a concomitant shift of the absorption maximum to 325 nm. The activity of the reduced enzyme is about 60% of the original one, and is not restored by dialysis.

*Molecular Weight* The molecular weight of the crystalline enzyme was examined in triplicate by the sedimentation equilibrium method. Assuming a partial specific volume of 0.74, a molecular weight of  $245,000 \pm 10,000$  was obtained.

*Structure of Subunit and NH<sub>2</sub>- and COOH-Terminal Residues*

The subunit composition of leucine dehydrogenase was determined by sodium lauryl sulfate polyacrylamide gel electrophoresis. The enzyme solution was dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) and then incubated with sodium lauryl sulfate (a final concentration, 1.0%) and 2-mercaptoethanol (0.1%) at 37° for 2 h. The treated enzyme, which catalytically inactive, was subjected to electrophoresis in the presence of 0.1% sodium lauryl sulfate and migrated as a single protein (Fig. 7). To determine the molecular weight of the polypeptide, I ran a series of standard proteins treated in the same manner: bovine serum albumin (Mr; 67,000), bovine liver glutamate dehydrogenase (Mr; 55,000),  $\gamma$ -globulin H-chain (Mr; 50,000), ovalbumin (Mr;

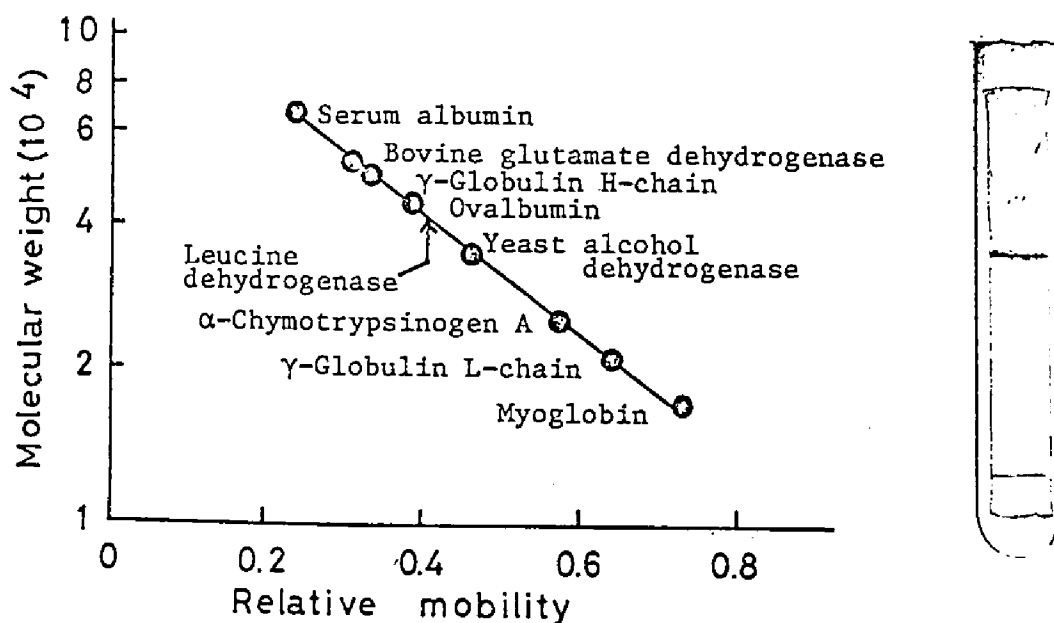


Fig. 7. Determination of the subunit molecular weight of leucine dehydrogenase by sodium lauryl sulfate disc gel electrophoresis. (left). Relationship between molecular weight and mobility. (right). Electrophoresis of the enzyme on sodium lauryl sulfate polyacrylamide gel.

43,000), yeast alcohol dehydrogenase (Mr; 37,000),  $\alpha$ -chymotrypsinogen A (Mr; 25,700),  $\gamma$ -globulin L-chain (Mr; 23,500) and myoglobin (Mr; 17,200). The molecular weight of leucine dehydrogenase was estimated to be about 41,000 from a semilogarithmic plot of molecular weight against mobility, showing that the enzyme consists of six subunits identical in molecular weight. Only methionine was identified as the  $\text{NH}_2$ -terminal amino acid of the enzyme, when analyzed by dansyl chloride method. The  $\text{COOH}$ -terminal amino acid was shown to be glutamate by tritium labeling method.

*Modification of Sulfhydryl Group of the Enzyme* Leucine dehydrogenase was inhibited with pCMB and  $\text{HgCl}_2$  as described in

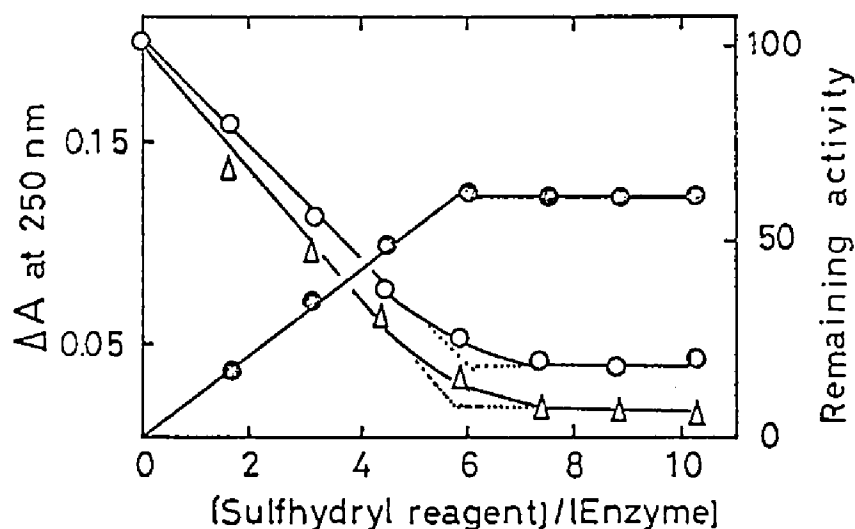


Fig. 8. Titration of sulfhydryl groups of native enzyme with pCMB and  $\text{HgCl}_2$ . (●): Spectrophotometric titration with pCMB (increase in absorbance at 250 nm); (○) activity after the titration with pCMB; (Δ) activity after the titration with  $\text{HgCl}_2$ . Enzyme concentrations:  $2.74 \mu\text{M}$  for pCMB titration,  $0.44 \mu\text{M}$  for  $\text{HgCl}_2$  titration. Incubation of the enzyme with sulfhydryl reagents was carried out for 90 min.



the previous section. The sulfhydryl groups, which are necessary for the catalytic activity, were titrated with several sulfhydryl reagents. Fig. 8 shows that titration of sulfhydryl groups of the enzyme with pCMB resulted in a linear decrease in activity and a linear increase in absorbance at 250 nm, with a concomitant decrease in activity. This indicates that a loss of activity is correlated with the modification of sulfhydryl groups by pCMB. The total number of sulfhydryl groups modified by pCMB was approximately 6 mol per mol of the enzyme. The remaining activity was about 18% of the native enzyme. The titration with  $\text{HgCl}_2$  also revealed the occurrence of 6 sulfhydryl groups per enzyme molecule, and the remaining activity was about 5% of the native enzyme. However, incubation of the enzyme with DTNB, 4-PDS or N-ethylmaleimide was not accompanied with a decrease in the enzyme activity. No increase in the absorbances at 412 nm and 324 nm was observed, when the enzyme was incubated with DTNB and 4-PDS, respectively.

Sulfhydryl groups of the enzyme denaturated by 8 M urea were titrated with DTNB, 4-PDS and pCMB. It was shown that 11.7-12.1 sulfhydryl groups per enzyme molecule reacted with the reagents in the presence of 8 M urea.

These results suggest that 2 sulfhydryl groups are found per subunit of the enzyme and one of them plays an essential role in the catalysis.

*Fluorometrical Studies on the Enzyme-NADH Binary Complex and the Determination of the Number of Binding Sites for NADH*  
The fluorescence of proteins is associated with their ultraviolet absorption with a major contribution of the tryptophan residue (44). When excited at 280 nm, leucine dehydrogenase showed a single emission band around 350 nm (Fig. 9). The fluorescence of the enzyme was quenched by forming complex

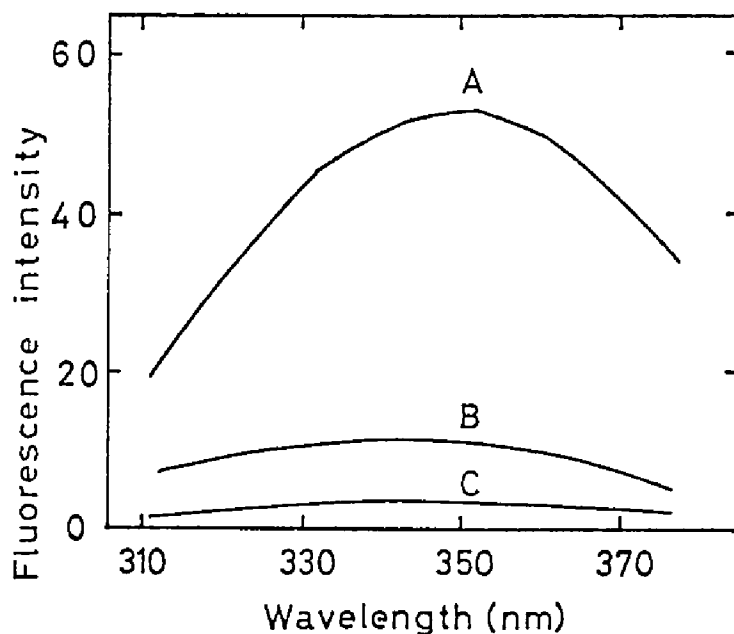


Fig. 9. Emission spectra of the enzyme and the enzyme-NADH complex. The emission spectra were measured in 0.1 M potassium phosphate buffer (pH 8.0). Curve A shows fluorescence of the enzyme (0.775  $\mu$ M), curve B, fluorescence of the enzyme (0.775  $\mu$ M) in the presence of NADH (25  $\mu$ M) and curve C, fluorescence of NADH (25  $\mu$ M). Excitation wavelength was 280 nm.

with NADH (Fig. 9).

On the other hand when excited at 340 nm the fluorescence spectrum of NADH showed the maximum at 450 nm (Fig. 10). The fluorescence at 450 nm of NADH increased by addition of the enzyme. However, no significant shift of emission maximum is observed. The similar findings have been observed for other nicotinamide-nucleotide dependent dehydrogenases (39-40, 45-46), and are utilized to follow NADH binding to the enzyme.

Leucine dehydrogenase was titrated with NADH by following fluorescence at 445 nm (Fig. 9-A). The maximum difference in NADH

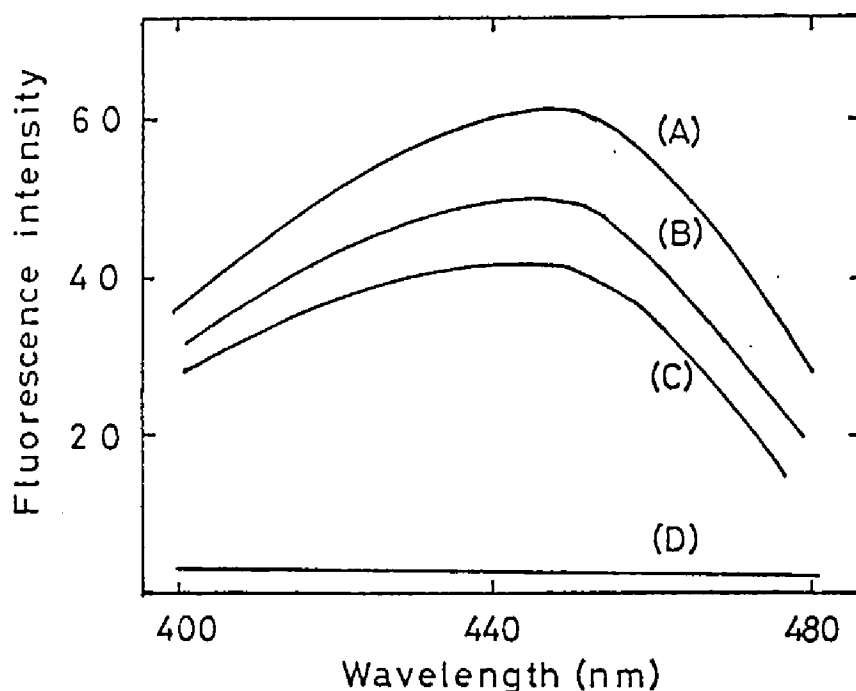


Fig. 10. Fluorescence of the enzyme-NADH complex.

Curve A shows the fluorescence emission spectrum of a mixture of the enzyme ( $1.47 \mu\text{M}$ ) and NADH ( $71 \mu\text{M}$ ); curve B, a mixture of the enzyme ( $0.74 \mu\text{M}$ ) and NADH ( $71 \mu\text{M}$ ); curve C, free NADH ( $71 \mu\text{M}$ ); and curve D, free enzyme ( $1.47 \mu\text{M}$ ). Excitation was at 340 nm.

fluorescence was estimated to be 16.7 from a double-reciprocal plot according to Prince and Radda (47). The data analysis according to Klotz leads to a straight line (Fig. 11-B), by extrapolating a value of 6 binding sites per hexamer was obtained. Dissociation constant ( $K_d$ ) obtained from reciprocal of the straight line was  $4.5 \mu\text{M}$ .

*Gel Filtration Studies of NADH Binding by Leucine Dehydrogenase* The binding of NADH was studied by gel filtration with Sephadex G-25. A typical elution profile is shown in Fig. 12. The results are summarized in Table VIII. Fig. 13 shows the results obtained by treating the data of Table III by the method of Hayes and

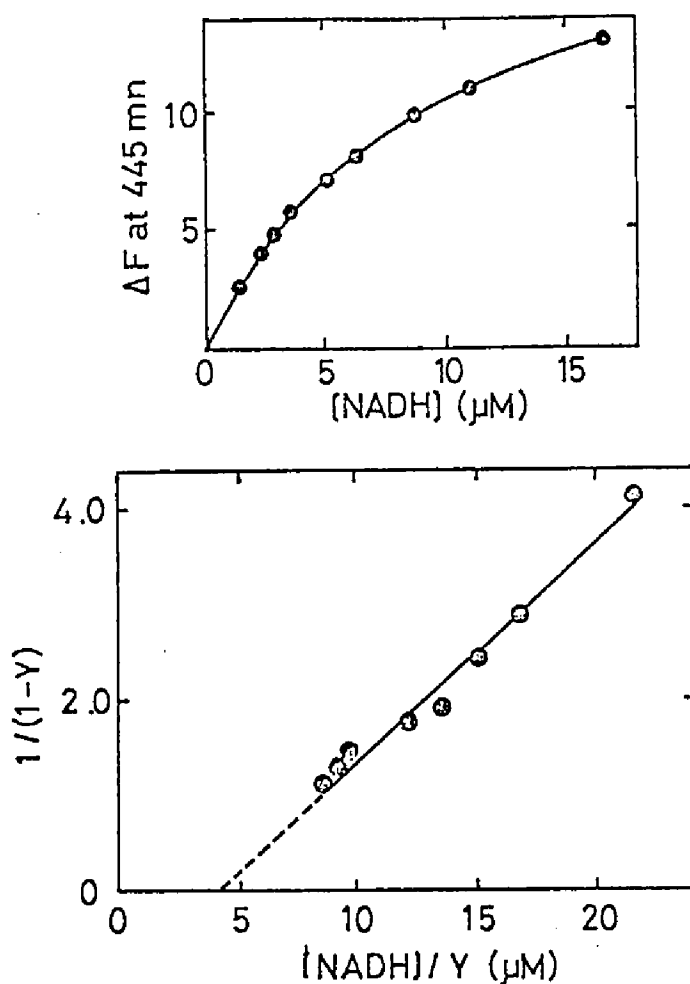


Fig. 11. Titration curve of the enzyme with NADH. (A) The enzyme solution ( $0.71 \mu M$ ) was titrated with NADH in  $0.1 M$  potassium phosphate buffer (pH 8.0). Nucleotide fluorescence was followed at 445 nm (excitation: 340 nm). (B) Klotz plots of titration curve. Extrapolation of the straight line gives the concentration  $n[E] = 4.2 \mu M$  of the binding sites.

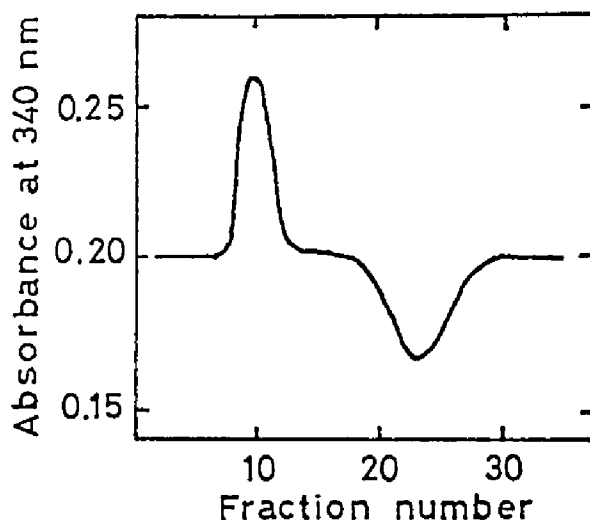


Fig. 12. Elution profile from gel filtration of NADH and the enzyme at pH 8.0 and 25°. The enzyme (6.26 nmol) was applied to the column (0.9 X 57 cm) equilibrated with 48  $\mu\text{M}$  NADH. Fractions of 1.4 ml were collected.

Table VIII. *Binding of NADH by leucine dehydrogenase*

Concen - tration of NADH ( $\mu\text{M}$ )	Enzyme added to column (nmol)	NADH bound calculated from trough fractions (nmol)	n	n/[NADH] ( $\mu\text{M}^{-1}$ )
22.5	3.82	19.30	5.05	0.224
32.2	6.82	36.83	5.40	0.168
48.0	6.26	35.00	5.59	0.116
61.9	3.67	21.37	5.82	0.094
82.3	6.53	39.36	6.03	0.079

Bound NADH was calculated from fractions constituting the trough in the elution profile using a molar absorption coefficient for NADH of  $6,220 \text{ M}^{-1}\text{cm}^{-1}$ .

Velick (48). The number of mol of NADH bound per mol of the enzyme ( $n$ ) was plotted against the quantity divided by the concentration of unbound NADH in the mixture ( $n/[NADH]$ ) (49). The intercept at the ordinate of Fig. 13. shows the binding of 6.3 mol of NADH per mol of the enzyme. The slope of the straight line is equal in magnitude to the dissociation constant of the enzyme-NADH complex which was estimated to be 5.5  $\mu$ M.

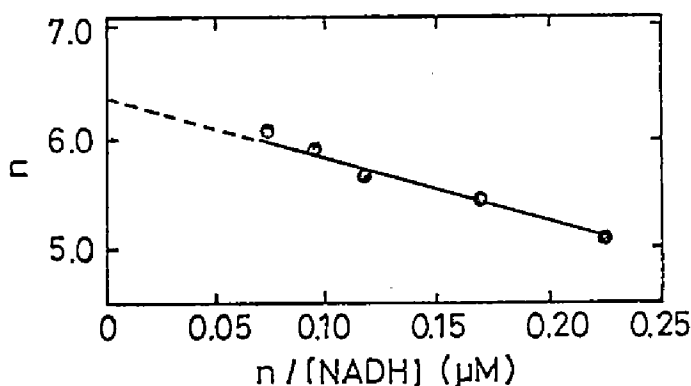


Fig. 13. Binding of NADH with the enzyme. The data of Table VIII were processed by the method of Hayes and Velick (48).

#### DISCUSSION

In this chapter, the physicochemical and enzymological properties of the crystalline leucine dehydrogenase were described. The enzyme has high pH optima for the oxidative deamination and the reductive amination and the difference in pH optima for the forward and reverse reactions is seen: pH optima are near pH 10.7 for the deamination and pH 9.0-9.5 for the amination. The differences in pH optima were shown for alanine dehydrogenase (14, 50)

and most glutamate dehydrogenases (3-4). This suggests that the activities of three amino acid dehydrogenases reflect the similar mechanisms.

The enzyme catalyzes the oxidative deamination of several branched and straight-chain L- $\alpha$ -amino acids except L-alanine, but other L-amino acids such as L-phenylalanine, L-glutamate and L-threonine, and D-amino acids are not the substrates. Replacement of  $\beta$  and in especial  $\gamma$ -hydrogen by methyl group results in enhancement of the reactivity. The length of a straight chain of L-amino acids also significantly influences the susceptibility to the enzyme: the C<sub>5</sub>, C<sub>4</sub> and C<sub>6</sub>-straight chain amino acids increase in the reactivity in this order. 4-Azaleucine and  $\gamma$ -methylallyl-glycine are very poor substrates, indicating that the substitution of  $\gamma$ -CH of leucine with nitrogen or the presence of a double bond in the side chain leads to a decrease in the rate of deamination. Sulfur analogues of norvaline and norleucine, in which  $\gamma$  and  $\delta$ -CH<sub>2</sub> were replaced, are less reactive substrates than the parent compounds. Either positively or negatively charged group in the side chain, e. g., hydroxyl,  $\epsilon$ -amino and  $\gamma$ -carboxyl groups probably prevents the amino acids from binding with the enzyme, because L-threonine, L-lysine and L-glutamate are neither substrates nor inhibitors for the deamination of L-leucine.

Hermier et al. (15) reported that leucine dehydrogenase from sporulating cells of *B. subtilis* deaminates L-alanine, and is inhibited competitively by D-alanine and D- $\alpha$ -aminobutyrate, and noncompetitively by D-leucine, D-valine and D-norvaline. Zink and Sanwall (9) reported that the enzyme from *B. subtilis* was not inhibited by D-leucine and D-valine. However, it was shown here that the *B. sphaericus* enzyme, which does not react with L-alanine, is not inhibited by D-alanine, but is competitively by D-enantiomers of the substrates.

$\text{NAD}^+$  is replaced by some of the  $\text{NAD}^+$ -analogues as a cofactor for leucine dehydrogenase as reported for several other dehydrogenases (51-53). 3-Acetylpyridine- $\text{NAD}^+$  is reduced by leucine dehydrogenase more rapidly than  $\text{NAD}^+$  as reported for bovine liver glutamate dehydrogenase and horse liver alcohol dehydrogenase (51). 3-Acetylpyridine-deamino  $\text{NAD}^+$  and deamino  $\text{NAD}^+$  have the closely similar reactivity to  $\text{NAD}^+$ . Thus, amino groups of nicotinamide and of adenine moiety of  $\text{NAD}^+$  are not of crucial importance for the coenzyme activity.

The enzyme is reversibly inhibited by pyridoxal 5'-phosphate. The inhibition is probably due to the formation of Schiff base between a lysine residue of the enzyme and 4-formyl group of pyridoxal 5'-phosphate as shown for glutamate dehydrogenase (3). This suggests that a lysine at or near the active site plays an important role in the catalytic action. In this regard, the enzyme is different from alanine dehydrogenase which is not affected by pyridoxal 5'-phosphate at all (54).

Mammalian glutamate dehydrogenases ( $\text{NAD(P)}^+$ ) are regulated by the purine nucleotides, e. g., inhibited by GTP and activated by ADP, but microbial glutamate dehydrogenases ( $\text{NAD}^+$  or  $\text{NADP}^+$ -specific) (3) and alanine dehydrogenase ( $\text{NAD}^+$ -specific) (54) are not affected, except  $\text{NAD}^+$ -specific glutamate dehydrogenase of *Thiobacillus novellus* (55), which is regulated by AMP. Leucine dehydrogenase of *B. sphaericus* also is unaffected by the purine nucleotides, suggesting that they are not concerned in control of the enzyme action. Thus, in general the amino acid dehydrogenases specific for only one of the two cofactors are not affected by the purine nucleotides, but glutamate dehydrogenases for which both  $\text{NAD}^+$  and  $\text{NADP}^+$  serve as a cofactor are regulated. The difference in regulation of the amino acid dehydrogenases by the nucleotides may reflect the difference in the physiological



functions of the enzymes.

Leucine dehydrogenase was strongly inhibited by pCMB (82%) and  $\text{HgCl}_2$  (95%), and the enzyme activity was lost by the modification of sulfhydryl groups. The activity was restored by excess mercaptans. This indicates that the reactive sulfhydryl groups participate in the catalytic activity, although their role is unknown. The enzyme was not affected by DTNB, 4-PDS and N-ethylmaleimide. Such difference of the sulfhydryl reagents in reactivity may reflect the difference in the environment of the sites involving the sulfhydryl groups.

The molecular weight of leucine dehydrogenase and the subunits are about 245,000 and 41,000, respectively. Methionine is the sole  $\text{NH}_2$ -terminal amino acid and glutamate is the sole  $\text{COOH}$ -terminal amino acid of the enzyme. This shows that the enzyme is a hexamer composed of identical subunits. The enzyme contains 6 reactive sulfhydryl groups and 6 coenzyme binding sites per molecule. This is also consistent with the hexameric structure composed of identical subunits and suggests the presence of one active site per subunit. A similar hexameric subunit structure has been reported for alanine dehydrogenases (54, 56) and many glutamate dehydrogenases except *Neurospora*  $\text{NAD}^+$ -dependent glutamate dehydrogenase (3). Many other dehydrogenases which act on the  $-\text{CH}-\text{OH}$  group of substrates, e. g., alcohol dehydrogenase, lactate dehydrogenase, glycer-aldehyde-3-phosphate dehydrogenase and malate dehydrogenase, are dimer or tetramer (57). Amino acid dehydrogenases are different from other dehydrogenases in this regard.

#### SUMMARY

Leucine dehydrogenase, which was purified to homogeneity and crystallized from *Bacillus sphaericus*, has a peak only at

280 nm and its  $A_{1\text{ cm}}^{1\%}$  is 9.18. The enzyme exhibits the optimum reactivity in alkaline pH for both forward and reverse reactions. The enzyme catalyzes the oxidative deamination of L-leucine, L-valine, L-isoleucine, L-norvaline, L- $\alpha$ -aminobutyrate and L-norleucine, and the reductive amination of their keto analogues. The enzyme requires  $\text{NAD}^+$  as a cofactor, which cannot be replaced by  $\text{NADP}^+$ . D-Enantiomers of the substrate amino acids inhibit competitively the oxidation of L-leucine. The enzyme activity is significantly reduced by sulfhydryl reagents and pyridoxal 5'-phosphate. Purine and pyrimidine bases, nucleosides and nucleotides have no effect on the activity. The molecular weight of the enzyme was determined to be about 245,000 by sedimentation equilibrium method. The enzyme consists of 6 identical subunits in molecular weight of about 41,000, and amino and carboxyl-terminal amino acid residues are methionine and glutamate, respectively. The enzyme contains 6 sulfhydryl groups required for catalysis and 6 coenzyme binding sites per enzyme molecule, suggesting the presence of one active site per subunit.

### CHAPTER III

THE KINETIC MECHANISM OF THE ENZYME REACTION AND THE STEREOSPECIFICITY OF THE HYDROGEN TRANSFER BETWEEN COENZYME AND SUBSTRATE CATALYZED BY LEUCINE DEHYDROGENASE.

In the preceding chapters, the purification and crystallization of leucine dehydrogenase from *B. sphaericus*, and the physicochemical and enzymological properties of the crystalline enzyme were described.

The present chapter describes the mechanism of the enzyme reaction by steady-state kinetic analysis and the stereospecificity of the enzymatic hydrogen transfer between the coenzyme and the substrate.

#### EXPERIMENTAL PROCEDURES

*Materials* NAD<sup>+</sup> and NADH were obtained from Kyowa Hakko Kogyo, Tokyo and amino acids were from Ajinomoto, Tokyo.  $\alpha$ -Keto acids were purchased from Sigma Chemical, bovine liver glutamate dehydrogenase from Boehringer Mannheim and DL-[2-<sup>3</sup>H]glutamate (9.5 mCi/mg) from New England Nuclear.

*Enzyme Preparation and Protein Determination* Crystalline leucine dehydrogenase was prepared from *B. sphaericus* as described in CHAPTER I. Alanine dehydrogenase was prepared from *B. sphaericus* (IFO 3525) (54). Protein determination was performed spectrophotometrically by measuring the absorbance at 250 nm ( $A_{1\text{ cm}}^{1\%} = 9.18$ ).

*Kinetic Measurements* The reaction was followed by measuring the appearance and disappearance of NADH at 340 nm using a Shimadzu MPS-50 L recording spectrophotometer. The standard incubation mixtures for the forward and reverse reactions are similar to those described in CHAPTERS I and II with some exceptions, which are specified in the figures.

Initial velocity and product inhibition experiments were carried out by varying the concentration of one substrate at different fixed level of the other substrate or the product.

The analysis of steady-state kinetic data and the nomenclature were carried out according to the methods proposed by Cleland (58-61). Michaelis constants were determined from the secondary plots of intercepts versus reciprocal concentrations of the varied substrate.

*Preparation of [4B-<sup>3</sup>H]NADH* The reaction mixture contained 224  $\mu\text{mol}$  of  $\text{NAD}^+$ , 0.072  $\mu\text{mol}$  of DL-[2-<sup>3</sup>H]glutamate (0.1 mCi), 0.3 mmol of L-glutamate, 4 mg of B-stereospecific bovine liver

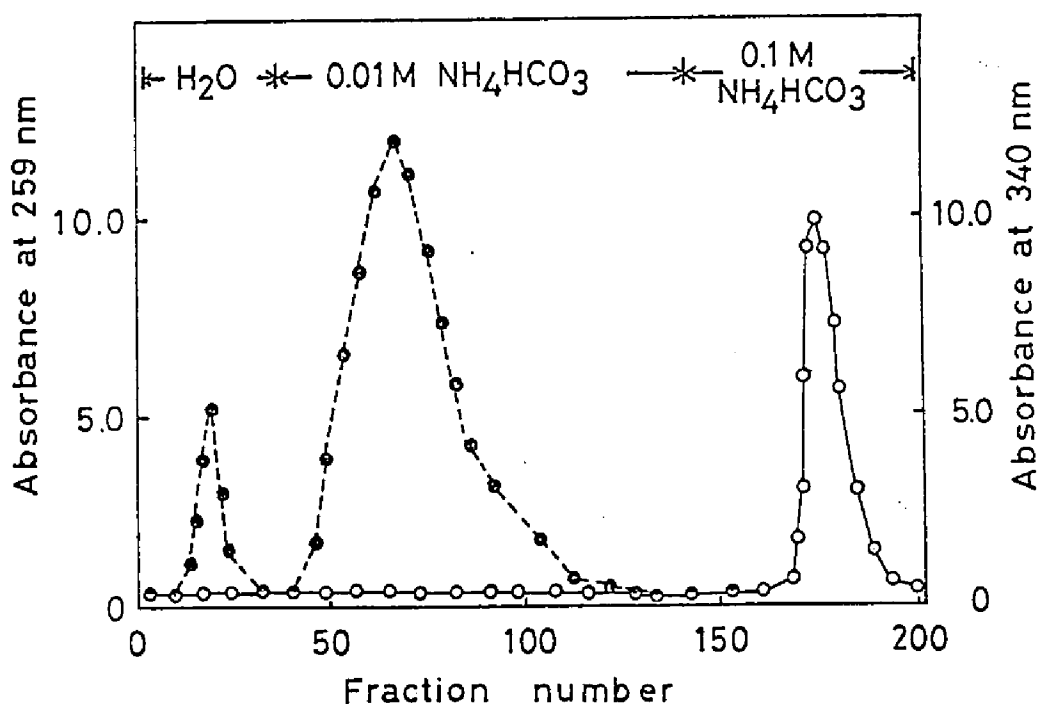


Fig. 14. Chromatography of [4B-<sup>3</sup>H]NADH on a column of DEAE-cellulose. Fractions of 2.8 ml were collected.  $\text{NAD}^+$  and NADH were monitored at 259 nm (●-●) and 340 nm (○-○), respectively.

glutamate dehydrogenase (120 units/mg) and 1.6 mmol of glycine-KCl-hydrazine hydrate buffer (pH 8.2) in a final volume of 8.5 ml. After incubation at 30° for 5 h, the reaction mixture was heated at 60° for 3 min, cooled and centrifuged. The supernatant solution was applied to a DEAE-cellulose column (bicarbonate form, 2.1 X 20 cm). After the column was washed with 0.01 M  $\text{NH}_4\text{HCO}_3$  to remove  $\text{NAD}^+$  unreacted, [4B- $^3\text{H}$ ]NADH was eluted with 0.1 M  $\text{NH}_4\text{HCO}_3$  (Fig. 14). Fractions containing [4B- $^3\text{H}$ ]NADH were combined and the specific activity was determined to be  $7.3 \times 10^7$  cpm/mmol.  $\text{NAD}^+$  and NADH were detected by following absorbances at 259 nm and 340 nm, respectively.

*Oxidation of [4B- $^3\text{H}$ ]NADH by Leucine Dehydrogenase or Alanine Dehydrogenase* The reaction mixture (10 ml) containing 10.3  $\mu\text{mol}$  of [4B- $^3\text{H}$ ]NADH, 100  $\mu\text{mol}$  of sodium  $\alpha$ -ketoisocaproate or sodium  $\alpha$ -ketobutyrate, 1.5 mmol of  $\text{NH}_4\text{Cl-NH}_4\text{OH}$  buffer (pH 9.5) and 0.91 mg of leucine dehydrogenase or 0.52 mg of alanine dehydrogenase was incubated at 30° for 2 h. After the reaction was stopped by adjusting the pH to 5 with 4 N-acetic acid, the mixture was chromatographed on a Dowex 1 X 8 column (formate form, 1.1 X 18 cm). Leucine and  $\alpha$ -aminobutyrate were determined with ninhydrin after paper chromatography according to the method as described previously (62). The amount of  $\text{NAD}^+$  was estimated as equal amount of leucine or  $\alpha$ -aminobutyrate formed.

*Counting of Radioactivity of Tritiated Compounds* Samples (0.5 ml or less) of radioactive solutions were mixed with 15 ml of a scintillation fluid consisting of naphthalene (50 g), 2,5-diphenyloxazole (7 g), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.6 g), ethanol (300 ml) and toluene to a final volume of 1 liter. The radioactivity was counted for 1 min in a Tri-Carb Liquid Scintillation Counter 3320.

## RESULTS

*Kinetic Mechanism* A series of steady state kinetic analyses were conducted to investigate the reaction mechanism of leucine dehydrogenase.

Initial velocity studies for the oxidative deamination reaction were first performed with  $\text{NAD}^+$  as a variable substrate in the presence of several fixed concentrations of L-leucine. Plots of reciprocals of initial velocities against reciprocals of  $\text{NAD}^+$  concentrations gave a family of straight lines which intersect in the upper left quadrant (Fig. 15-A). The apparent Michaelis constant for one substrate is dependent on the concentration of the other. According to Cleland (58), the

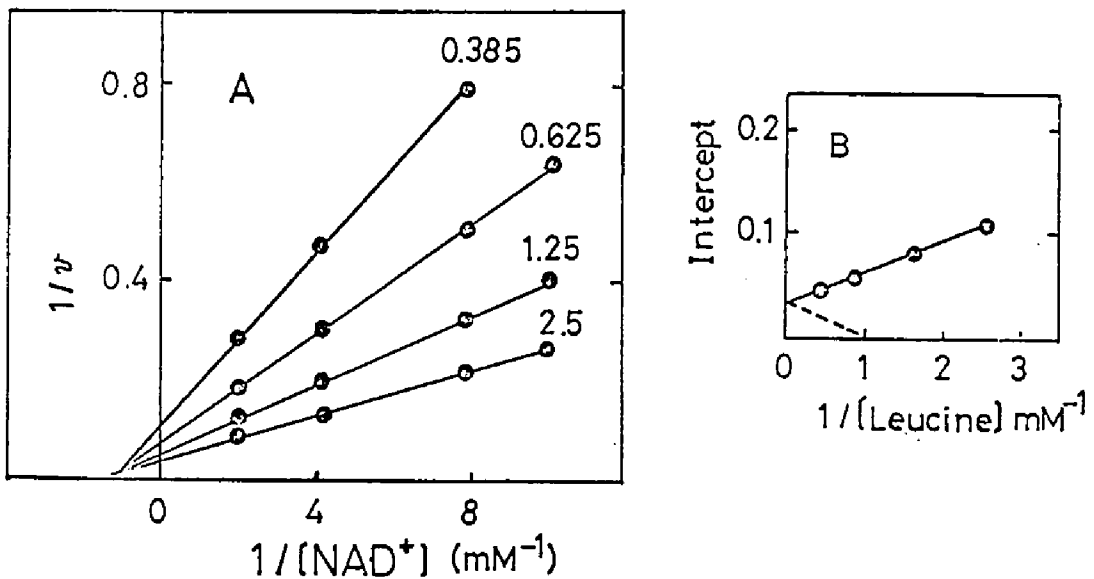


Fig. 15. Double reciprocal plots of initial velocity against  $\text{NAD}^+$  concentrations at a series fixed concentrations of leucine .  
(A). Leucine concentrations (mM) are indicated in the figure.  
(B): Secondary plot from the intercepts against the fixed leucine concentrations.

data shown in Fig. 15-A indicate that the reaction proceeds via the formation of ternary complex of the enzyme with  $\text{NAD}^+$  and leucine, both of which bind to the enzyme before release of the products. The Michaelis constants for  $\text{NAD}^+$  and leucine were calculated to be 0.39 and 1 mM, respectively, from the secondary plots of intercepts *versus* reciprocal of the other non-varied substrate concentration (Fig. 15-B) (58, 61).

A kinetic analysis in the reductive amination reaction was performed to distinguish several reaction mechanisms (58, 61). Fig. 16-A shows double reciprocal plots of velocities against  $\alpha$ -ketoisocaproate concentrations at several fixed concentrations of ammonia and a high and constant concentration of NADH. The double reciprocal plots gave straight lines intersecting on the abscissa. At a high level of ammonia, the double reciprocal plots of velocities against NADH concentrations at several fixed concentrations of  $\alpha$ -ketoisocaproate also gave straight intersecting lines (Fig. 16-C). However, with  $\alpha$ -ketoisocaproate at a saturating concentration, the double reciprocal plots of velocities against NADH concentrations at several different concentrations of ammonia were quite different from those shown in Figs. 16-A and 16-C, and gave parallel lines (Fig. 16-B). These observed kinetic patterns rule out the possibility of random addition of substrates and represent a sequential ordered mechanism in which  $\alpha$ -ketoisocaproate binds to the enzyme between NADH and ammonia. The Michaelis constants for NADH,  $\alpha$ -ketoisocaproate and ammonia were calculated to be 35  $\mu\text{M}$ , 0.31 mM and 0.2 M, respectively.

The product inhibition studies on the reductive amination reaction were further carried out to determine the order of substrate addition and product release according to the method of Cleland (58, 61). With  $\text{NAD}^+$  as an inhibitor, the double reciprocal plots of velocities against NADH concentrations at  $\uparrow$

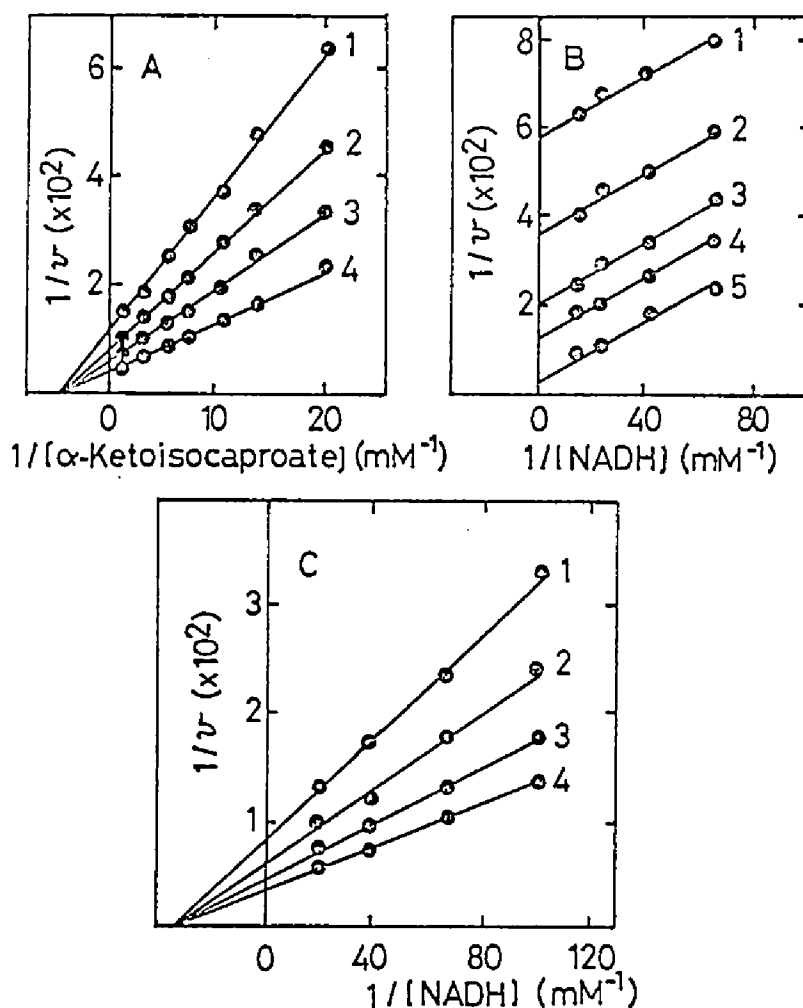


Fig. 16. Initial velocity patterns for the reductive amination reaction. A: Double reciprocal plots of velocity against  $\alpha$ -ketoisocaproate concentrations at several fixed concentrations of ammonia in the presence of a high concentration of NADH (0.23 mM). The concentrations of ammonia used were: 1, 100 mM; 2, 150 mM, 3; 250 mM; and 4, 500 mM. B: Double reciprocal plots of velocity against NADH concentrations at several fixed concentrations of ammonia in the presence of a high concentration of  $\alpha$ -ketoisocaproate (10 mM). The concentrations of ammonia used



were: 1, 20 mM; 2, 30 mM; 3, 60 mM; 4, 100 mM; and 5, 200 mM. C: Double reciprocal plots of velocity against NADH concentrations at several fixed concentrations of  $\alpha$ -ketoisocaproate in the presence of a high concentration of ammonia (750 mM). The concentrations of  $\alpha$ -ketoisocaproate used were: 1, 0.15 mM; 2, 0.25 mM; 3, 0.5 mM; and 4, 1.0 mM.

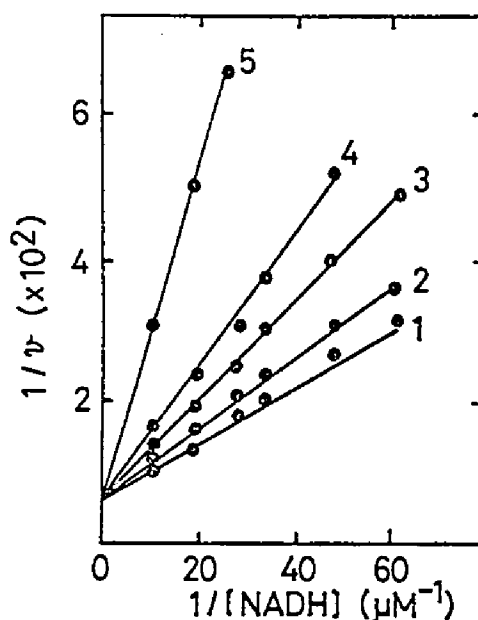


Fig. 17. Product inhibition by  $NAD^+$  with NADH as the variable substrate. Ammonia (750 mM) and  $\alpha$ -ketoisocaproate (10 mM) were present at constant high concentration. The concentrations of  $NAD^+$  used were: 1, 0; 2, 0.1 mM; 3, 0.3 mM; 4, 0.5 mM; and 5, 1.0 mM.

Table IX. *Predicted and observed product inhibition patterns ordered ternary-binary mechanism.*

Varied substrates	Inhibitors <sup>a</sup> and inhibition patterns <sup>b</sup>			
	NAD <sup>+</sup>		L-Leucine	
	Predicted	Observed	Predicted	Observed
NADH <sup>c</sup>	Com	Com	Un	Un
$\alpha$ -Ketoisocaproate <sup>d</sup>	Un	Un	Un	Un
Ammonia <sup>e</sup>	Un	Un	Non	Non

<sup>a</sup> The concentrations of NAD<sup>+</sup> and leucine used were 0, 0.1, 0.3, 0.5 and 1.0 mM, and 0, 1, 2.5, 5 and 10 mM, respectively.

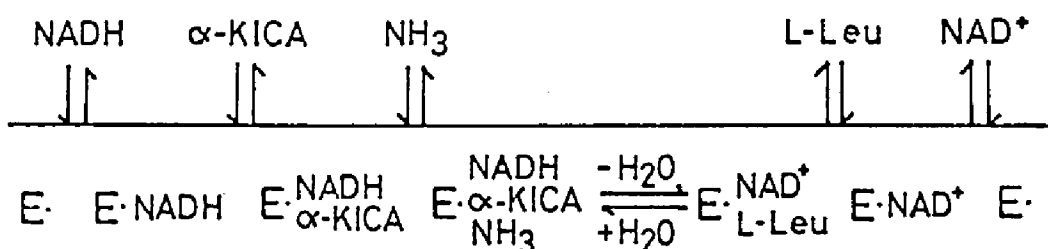
<sup>b</sup> The abbreviations used: Com, competitive; Un, uncompetitive; and Non, noncompetitive. Predicted product inhibition patterns were obtained from the steady state algebraic equation for the sequential ordered ternary-binary kinetic mechanism proposed by Cleland (58).

<sup>c</sup> The concentrations of NADH: 16, 20, 30, 37, 52, and 111  $\mu$ M,  $\alpha$ -ketoisocaproate: 10 mM, and ammonia: 750 mM.

<sup>d</sup> The concentrations of  $\alpha$ -ketoisocaproate: 0.1, 0.2, 0.3, 0.5, 0.75, 1.0 and 1.5 mM, NADH: 293  $\mu$ M, and ammonia: 750 mM.

<sup>e</sup> The concentrations of ammonia: 50, 80, 100, 200, 300 and 500 mM, NADH: 293  $\mu$ M, and  $\alpha$ -ketoisocaproate: 10 mM.

† constant high concentrations of  $\alpha$ -ketoisocaproate and ammonia showed competitive inhibition (Fig. 17). These findings suggest that NADH and  $\text{NAD}^+$  can bind to the free form of enzyme and thus NADH binds first to the enzyme, followed by  $\alpha$ -ketoisocaproate and then ammonia, and also that  $\text{NAD}^+$  is the last product that is released from the enzyme. As shown in Table IX, the other product inhibition patterns observed with  $\text{NAD}^+$  and L-leucine as the inhibitors are identical with the predicted patterns for the sequential ordered ternary-binary kinetic mechanism. The observation of noncompetitive inhibition by L-leucine with respect to ammonia rules out a mechanism of Theorell-Chance type (63). From these results, the sequence of addition of the substrates in the reductive amination reaction is NADH,  $\alpha$ -ketoisocaproate and ammonia, and that of release of products is leucine and  $\text{NAD}^+$  as shown in the following scheme:



E: Enzyme,  $\alpha$ -KICA:  $\alpha$ -Ketoisocaproate, L-Leu: L-Leucine.

*Stereospecificity of Hydrogen Transfer between Coenzyme and Substrate* When  $\alpha$ -ketoisocaproate was reduced to leucine in the presence of stereospecifically labeled NADH, [4B- $^3\text{H}$ ]NADH, and  $\text{NAD}^+$  and leucine formed were separated by Dowex 1 X 8 chromatography (Fig. 18), substantially all radioactivity of [4B- $^3\text{H}$ ]NADH was transferred to leucine but not retained in  $\text{NAD}^+$  (Table X). This incorporation of radioactivity into leucine †

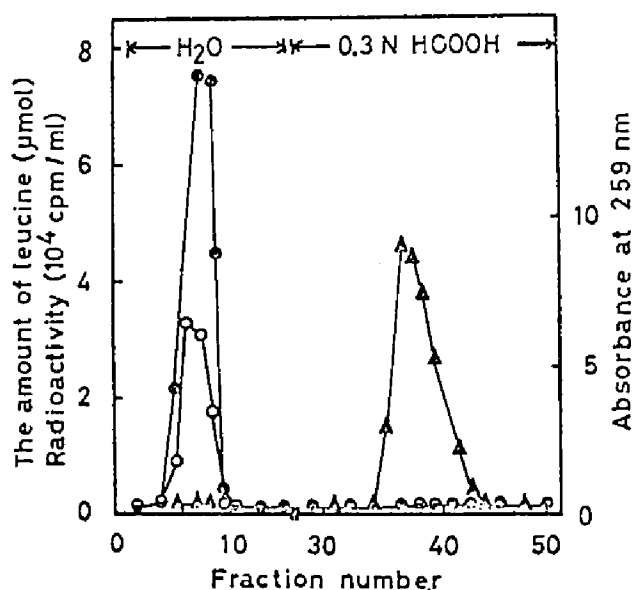


Fig. 18. Chromatography on a column of Dowex 1 X 8 (formate form) of leucine and NAD<sup>+</sup>. Fractions of 2.8 ml were collected. ▲, Absorbance at 259 nm; o, the amount of leucine; ●, radioactivity.

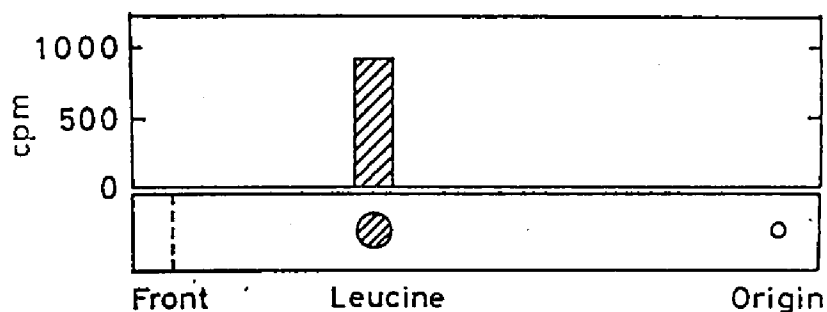


Fig. 19. Incorporation of tritium into leucine. Fraction number 7 (0.1 ml) in Fig. 18 was chromatographed on Toyo No. 53 filter paper, using *n*-butanol-acetic acid-water (12:3:5) as a solvent. After the development, the paper strip (2.5 X 20 cm) was cut out into serial 1.2 cm sections. They were transferred to scintillation vials containing the scintillation fluid and the radioactivity was measured.

Table X. Stereospecificity of hydrogen transfer of [4B-<sup>3</sup>H]NADH to leucine and α-aminobutyrate.

Enzymes (Substrates used)	Radioactivity (10 <sup>7</sup> cpm/mmol)			
	Before reaction	After reaction		
	[4B- <sup>3</sup> H]NADH	NAD <sup>+</sup>	L-Leucine	L-α-Amino- butyrate
Leucine dehydrogenase (α-Ketoisocaproate)	7.28 (100%)	0.32 (4.4%)	7.05 (97%)	- -
Leucine dehydrogenase (α-Ketobutyrate)	7.56 (100%)	0.50 (6.6%)	- -	7.90 (104%)
Alanine dehydrogenase (α-Ketobutyrate)	7.32 (100%)	6.81 (93%)	- -	0.46 (6.0%)

† was confirmed by paper chromatography of the solution eluted with water (Fig. 19). These results indicate that *pro-S* hydrogen at C-4 of the dihydronicotinamide ring of NADH is exclusively transferred to the substrate by the enzyme without exchange with protons of the medium: the enzyme is B-stereospecific.

On the other hand, alanine dehydrogenase isolated from *B. sphaericus* (54) was shown to be A-stereospecific as reported for the enzyme of *B. subtilis* (64). Thus, leucine and alanine dehydrogenases from *B. sphaericus* show the opposite stereospecificity. The author reconfirmed their stereospecificity with [4B-<sup>3</sup>H]NADH and sodium α-ketobutyrate which is the common substrate of both enzymes. As shown in Table X, the tritium of

[4B-<sup>3</sup>H]NADH was transferred to  $\alpha$ -aminobutyrate with leucine dehydrogenase, but essentially all the radioactivity remained in NAD<sup>+</sup> with alanine dehydrogenase. These results show that even when leucine and alanine dehydrogenases catalyze the reductive amination of the same  $\alpha$ -keto acid, the former transfers the *pro-S* hydrogen at C-4 of dihydronicotinamide ring to the substrate and the latter does the *pro-R* hydrogen.

#### DISCUSSION

The reductive amination of leucine dehydrogenase is a ter-reactants and bi-products reaction. The steady state kinetic studies revealed the reaction to be a sequential ordered ternary-binary mechanism. The similar mechanism was reported for the several ter-reactants nicotinamide nucleotide-linked dehydrogenases: saccharopine dehydrogenase (65), octopine dehydrogenase (66) and pigeon malic enzyme (67). On the other hand, two different kinetic mechanisms have been reported for glutamate dehydrogenase. Bovine liver glutamate dehydrogenase catalyzes the reaction in a random ordered mechanism (68-70), and the enzyme from pig heart mitochondria (71), microorganisms (72-74) and plant (75) catalyze the reaction through a sequential ordered mechanism. Various other mechanisms, i, e., sequential ordered (76), ordered random (77) and ping pong mechanism (78), are reported for glyceraldehyde 3-phosphate dehydrogenase. However, the significance of the discrepancy in the mechanism of dehydrogenation is not known at present.

Nicotinamide nucleotide-linked dehydrogenases show A or B-stereospecificity for hydrogen removal from the C-4 position of the nicotinamide moiety of the reduced coenzyme (79-81). The stereospecificity has been determined for a large number of dehydrogenases (80-81), but the stereospecificity for

dehydrogenases whose dehydrogenation occurs at -C-N- system were examined with only a few enzymes. Glutamate dehydrogenases (82-83) and octopine dehydrogenase (84) were shown to be B-specific. Alizade et al. (64) reported that alanine dehydrogenase from *B. subtilis* is A-specific; the *pro-R* hydrogen at C-4 of the reduced nicotinamide ring is transferred to pyruvate, and proposed that  $\text{NAD}^+$ -linked dehydrogenases working on amino group of non-phosphorylated substrates belong to the stereospecific "A class". This, however, is not the case for leucine dehydrogenase which is B-stereospecific as shown here. The stereospecificity of hydrogen transfer is characteristic of the enzyme, but not of the substrate, because leucine dehydrogenase and alanine dehydrogenase reactions proceed with B and A-stereospecificity, respectively, also in the reaction system containing  $\alpha$ -ketobutyrate, a common substrate.

#### SUMMARY

The reductive amination catalyzed by leucine dehydrogenase is a three-substrates and two-products reaction. The results obtained from initial velocity and product inhibition studies are consistent with a sequential ordered ternary-binary mechanism where NADH binds first to the enzyme followed by  $\alpha$ -ketoisocaproate and then ammonia, and the products are released in the order of L-leucine and  $\text{NAD}^+$ . The Michaelis constants are as follows: L-leucine (1 mM),  $\text{NAD}^+$  (0.39 mM), NADH (35  $\mu\text{M}$ ),  $\alpha$ -ketoisocaproate (0.31 mM) and ammonia (0.2 M).

Stereospecificity of the enzymatic hydrogen transfer between the coenzyme and the substrate was investigated. The *pro-S* hydrogen at C-4 of the dihydronicotinamide ring of NADH is exclusively transferred to the substrate; the enzyme is B-stereospecific.

## CHAPTER IV

### THE APPLICATIONS OF LEUCINE DEHYDROGENASE

#### [A] ANTINEOPLASTIC ACTIVITY

Some observations on the inhibition of tumor growth by enzymes have stimulated the search for the antineoplastic activity of other microbial enzymes related to amino acid metabolism. Most of the antitumor enzymes, e. g., asparaginase (85-86), glutaminase (87), glutaminase-asparaginase (88), arginase (89-90) and phenylalanine ammonia-lyase (91) catalyze the essentially irreversible degradation of amino acids. The antitumor activities of a bacterial folate-cleaving enzyme, carboxypeptidase G<sub>1</sub> (92-93) and jack bean urease (94), and the inhibition of growth and DNA synthesis of tumor cells by ascorbic acid oxidase (95) also have been reported in recent years.

In the present section, studies on the antineoplastic activity of leucine dehydrogenase from *B. sphaericus* are described.

#### EXPERIMENTAL PROCEDURES

Leucine dehydrogenase and alanine dehydrogenase (54) purified from *B. sphaericus* and bovine liver glutamate dehydrogenase (type II) purchased from Boehringer Mannheim were employed. The enzymes were dialyzed overnight against 500 volume of 0.01 M sodium phosphate buffer (pH 7.6) containing 0.9% NaCl.

The therapeutic experiments were carried out with the Ehrlich ascites carcinoma carried in DD mice. Ehrlich ascites carcinoma cells were inoculated by intraperitoneal injection of 0.5 ml aliquots containing  $2 \times 10^6$  cells in 20 to 22 g DD mice. The enzymes were injected intraperitoneally once daily for 10 to 14 days consecutively, starting 24 h after tumor inoculation.



Antitumor activity was evaluated by the increase in life span and weight gain from tumor growth.

## RESULTS

Leucine dehydrogenase was shown to be highly inhibitory to Ehrlich ascites carcinoma *in vivo* (Fig. 20). Tumor-bearing mice treated with leucine dehydrogenase showed a progressive increase in life span with increasing doses of the enzyme (Fig. 20). The results of antitumor activity are summarized in Table XI. The dosage of 4 mg/kg X 10 to 14 days produced 236 to 300% increase in mean survival time over controls, and smaller doses still provided increased mean survival time: 219 to 170% at 1 mg/kg X 14 days, and 136 and 191% at 2 mg/kg X 10 and 14 days. Furthermore, cures (60-day survivors) were

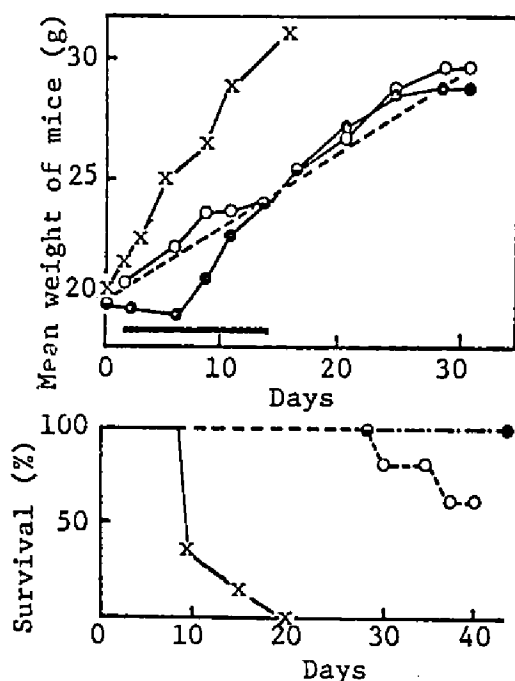


Fig. 20. Inhibition of the growth of Ehrlich ascites carcinoma by leucine dehydrogenase.

Intraperitoneal treatment with leucine dehydrogenase (—) was started 24 h later. (----): control mice, (x): tumor-bearing mice, (o): tumor-bearing mice treated with 1 mg/kg/day of the enzyme, (●) tumor-bearing mice treated with 4 mg/kg/day of the enzyme.

Table XI. Antitumor activity of purified enzymes and  $\alpha$ -ketoisocaproate

	Dose X day (mg/kg/day)	Body weight increased on 14 th day (g)		Mean survival day of mice (range)		Treated Control (%)	Treated 30 or 60 days survivors	
		Control	Treated	Control	Treated		Control	Treated
Leucine dehydrogenase	(1) 1 X 14	+ 8.3	+ 4.2	13.6(10-20)	29.8(29-41+)	219	0/5	4/5 *
	4 X 14	+ 8.3	+ 4.2	13.6(10-20)	41 ( 41+)	300	0/5	4/5 *
	(2) 2 X 10	+ 11.5	+ 10.0	17.9(12-21)	24.4(14-54+)	136	0/10	1/5 *
.	4 X 10	+ 11.5	+ 5.7	17.9(12-21)	42.4(14-54+)	236	0/10	3/5 *
	8 X 10	+ 11.5	+ 5.6	17.9(12-21)	32.2(17-54+)	180	0/10	2/5 *
	(3) 1 X 14	+ 14.1	+ 7.3	15.3(11-17)	26.0(18-46+)	170	0/10	1/5 *
$\alpha$ -Ketoiso- caproate	2 X 10	+ 14.1	+ 5.0	15.3(11-17)	29.2(18-46+)	191	0/10	2/5 *
	5 X 12	+ 14.1	+ 11.1	15.3(11-17)	20.1(13-30+)	131	0/5	2/5 **
	10 X 12	+ 14.1	+ 14.3	15.3(11-17)	16.4(13-22)	107	0/5	0/5 **
Glutamate dehydrogenase	50 X 14	+ 14.1	+ 8.3	15.3(11-17)	14.6(8-23)	96	0/5	0/5 **
	2 X 14	+ 14.1	+ 14.0	15.3(11-17)	16.6(9-18)	108	0/5	0/5 **
	5 X 14	+ 14.1	+ 12.1	15.3(11-17)	19.4(9-28)	127	0/5	0/5 **
Alanine dehydrogenase	10 X 14	+ 14.1	+ 13.8	15.3(11-17)	14.6(11-30+)	120	0/5	1/5 **
	2 X 14	+ 14.1	+ 16.0	15.3(11-17)	15.6(9-24)	102	0/5	0/5 **
	10 X 14	+ 14.1	+ 15.5	15.3(11-17)	16.6(11-26)	108	0/5	0/5 **

\* Treated 60 days survivors. \*\* Treated 30 days survivors.

observed in 5 out of 10 mice at 1 mg/kg X 14 days, 2 out of 10 mice at 2 mg/kg X 10 to 14 days, and 7 out of 10 mice at 4 mg/kg X 10 to 14 days of treatment with leucine dehydrogenase. Neither  $\alpha$ -ketoisocaproate, which is a product of the oxidative deamination of leucine, bovine liver glutamate dehydrogenase, nor *B. sphaericus* alanine dehydrogenase inhibited the growth of Ehrlich ascites carcinoma cells *in vivo*.

#### DISCUSSION

Leucine dehydrogenase showed the high activity to inhibit the growth of Ehrlich ascites carcinoma *in vivo*. Leucine dehydrogenase is the first antitumor enzyme as a dehydrogenase. The antineoplastic activity of enzymes such as asparaginase (96-97) and glutaminase (87) is probably ascribable to enzymatic depletion of the amino acids required for the growth of certain human lymphoblastic leukemias.

The antitumor activities of carboxypeptidase G<sub>1</sub> also was proved to be due to in creating rapid folate depletion (92). The antitumor activity of leucine dehydrogenase also may be explained in a similar way: the enzyme reduces the blood concentration of L-branched-chain amino acids, essential amino acids of cells, to inhibit the growth. Leucine dehydrogenase showed the inhibitory effect on the incorporation of <sup>14</sup>C-leucine in the presence of NAD<sup>+</sup>. This result supports the above possibility. The parenteral administration of enzymes which degrade amino acids required for growth of neoplasms offers a potential cancer therapy with marked specificity for the tumor (98). Leucine dehydrogenase also produced high anti-neoplastic effect in case of Ehrlich ascites carcinoma, but had no therapeutic effectiveness against other neoplasms, sarcoma 180 and 6C3HED·OG. This specificity may result from

a difference in requirement by different tumor cells for leucine and other substrate amino acids. Further experiments, however, are required to elucidate the mechanism of antitumor, e. g., the relationship between the enzymological and physiological functions and antitumor activity of the enzyme (99).

#### SUMMARY

Leucine dehydrogenase from *Bacillus sphaericus* showed the antitumor activity against Ehrlich ascites carcinoma in DD mice.

#### [B] DETERMINATION OF L-BRANCHED-CHAIN AMINO ACIDS AND THEIR KETO ANALOGUES WITH LEUCINE DEHYDROGENASE

L-Leucine, L-valine and L-isoleucine are metabolically important as essential amino acids in mammals, and as well as their keto analogues are responsible for the disorders of the branched-chain amino acids metabolism such as maple syrup disease (100). The specific and simple method of determination of the branched-chain amino acids and their keto analogues is required in clinical chemistry, food chemistry and other related fields.

In this section, the methods for the determination of L-branched-chain amino acids and their keto analogues with leucine dehydrogenase are described.

#### EXPERIMENTAL PROCEDURES

*Materials* NAD<sup>+</sup> and NADH were obtained from Kyowa Hakko Kogyo, Tokyo, amino acids from Ajinomoto, Tokyo, and  $\alpha$ -keto acids from Sigma Chemical. Iodonitrotetrazolium chloride (INT), nitrobluetetrazolium chloride (Nitro-TB), thiazolyl blue (MTT) and phenazine methosulfate (PMS) were purchased from Nakarai Chemicals, Kyoto, Japan. Leucine dehydrogenase was prepared as described in CHAPTER I. Other chemicals used were of analytical

grade. Spectrophotometric measurements were performed with a Carl Zeiss PMQ II spectrophotometer or a Shimadzu MPS-50L recording spectrophotometer.

*Determination of L-Branched-Chain Amino Acids* L-Branched-chain amino acids were determined spectrophotometrically by measuring an increase of the absorbance at 340 nm using the following reaction mixture (0.8 ml): 70  $\mu\text{mol}$  of glycine-KCl-KOH buffer (pH 10.7), 10  $\mu\text{mol}$  of  $\text{NAD}^+$ , L-branched-chain amino acid (0.3 ml) and 5-10  $\mu\text{g}$  of leucine dehydrogenase (0.05 ml). In a blank, water was substituted for the amino acid. The reaction mixture was incubated at 25° for 30 min.

The colorimetric determination of L-branched-chain amino acids includes coupling NADH formation with the reduction of a tetrazolium salt. PMS was used as an intermediate electron carrier. The reaction mixture (1 ml) in a colored test tube contained 1.5  $\mu\text{mol}$  of  $\text{NAD}^+$ , 0.4  $\mu\text{mol}$  of INT, 0.16  $\mu\text{mol}$  of PMS and 40-70  $\mu\text{g}$  of leucine dehydrogenase (0.1 ml), L-branched-chain amino acid (0.1 ml) and 110  $\mu\text{mol}$  of glycine-KCl-KOH buffer (pH 9.0). The reaction was initiated by addition of the color reagent which consists of  $\text{NAD}^+$ , INT and PMS. The reaction mixture was incubated at 37° and, at a suitable interval, the reaction was arrested by addition of 0.5 N-HCl (1 ml). The mixture was shaken with *tert*-amylalcohol (2 ml) to extract the hormazan formed. After centrifugation (3,000 rpm for 5 min), 1 ml of the colored *tert*-amylalcohol layer (upper) was added to 0.2 ml of ethyl cellosolve and mixed, and the absorbance at 490 nm was measured.

*Determination of L-Branched-Chain Keto Acids* Branched-chain  $\alpha$ -keto acids were determined spectrophotometrically by measuring a decrease of the absorbance at 340 nm due to the disappearance of NADH using the following reaction mixture (1.0 ml): 650  $\mu\text{mol}$  of  $\text{NH}_4\text{Cl}$ - $\text{NH}_4\text{OH}$  buffer (pH 9.5), 0.2  $\mu\text{mol}$  of

NADH, keto acid (0.2 ml) and 1-5  $\mu$ g of leucine dehydrogenase (0.05 ml). The reaction was initiated by addition of the enzyme solution and the mixture was incubated 25° for 30 min.

## RESULTS AND DISCUSSION

*Spectrophotometric Determination of L-Branched-Chain Amino Acids* The calibration curve for L-leucine is shown in Fig. 21. A linear relationship between the absorbance at 340 nm and the amount of L-leucine was obtained in the range of 0.02 to 0.2  $\mu$ mol of L-leucine. L-Valine and L-isoleucine could be also determined in the same way. D-Leucine (0.1 mM), which is one of the strongest competitive inhibitors, did not interfere with the L-leucine determination.

### *Colorimetric Determination of L-Branched-Chain Amino Acids*

L-Branched-chain amino acids were determined by coupling the leucine dehydrogenase reaction with the reduction of a tetrazolium salt. The assay system contains PMS as a primary electron acceptor and a tetrazolium salt as a terminal oxidant

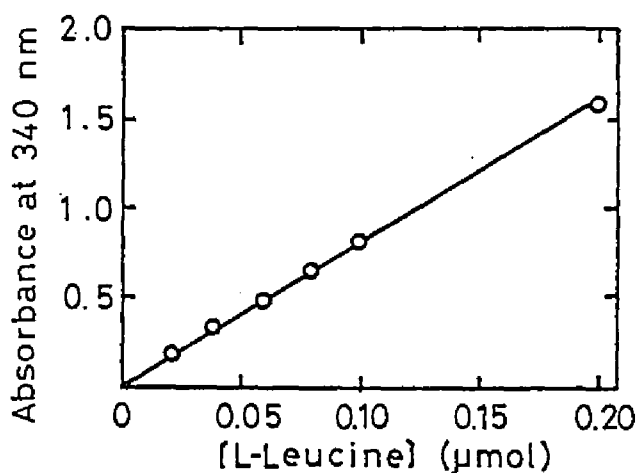


Fig. 21. Calibration curve for L-leucine.

to transfer the electron of NADH. INT possesses only a slight solubility in water when transferred to the reduced state, formazan (101), and is not applicable to the accurate measurement. Attempts were made to extract the formazan with various organic solvent. The reaction mixture was shaken with one of ethanol, toluene, chloroform, pyridine, N,N-dimethylformamide, *iso*-amylalcohol and *tert*-amylalcohol. The formazan was successfully extracted with *tert*-amylalcohol. The formazan solution in *tert*-amylalcohol was clear red (absorption maximum: 490 nm) and stable for at least several hours, but became turbid when transferred into a quartz cell. The addition of ethyl cellosolve to the solution prevented appearance of turbidity.

Though the optimum pH for the oxidative deamination is around 10.7 (CHAPTER II), the determination of L-branched-chain amino acids was carried out at pH 9.0 to avoid decomposition of the color reagent. Since the reaction was finished substantially within about 15 min, the absorbance at 490 nm was measured after 20 min. Under the conditions a linear relationship was obtained

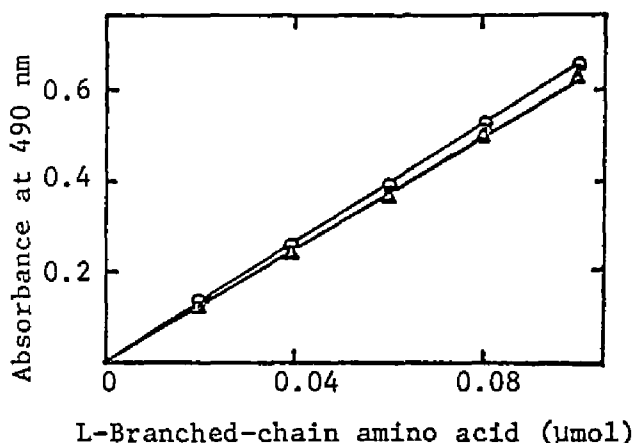


Fig. 22. Calibration curves for L-leucine (o-o), L-valine ( $\bullet$ - $\bullet$ ) and L-isoleucine ( $\blacktriangle$ - $\blacktriangle$ ).

between the absorbance at 490 nm and the amount of L-leucine, L-valine or L-isoleucine ranging from 0.02 to 0.10  $\mu\text{mol}$  (Fig. 22).

D-Branched-chain amino acids, which are the competitive inhibitors of leucine dehydrogenase, did not interfere with the determination of L-leucine.

The formazans with Nitro-TB and MTT have higher molar absorption coefficients than with INT, but both formazans were less sufficiently extracted with *tert*-amylalcohol than that with INT (Fig. 23).

Since PMS is labile to light and oxygen (101), Merdola blue, which is practically insensitive to light and shows a somewhat higher rate of electron transfer (101), was used as a primary electron acceptor without success because of unstability of Merdola blue in alkali.

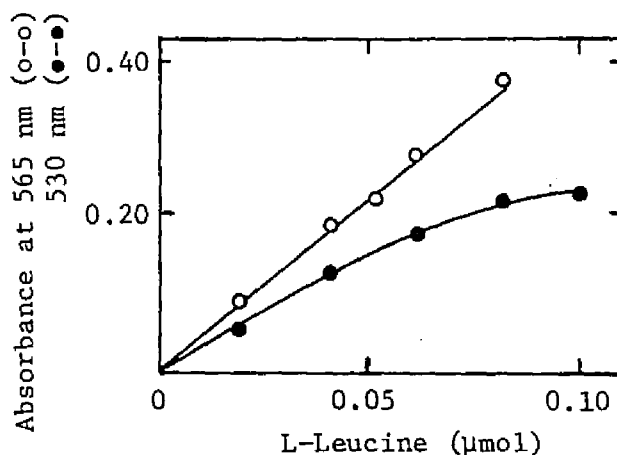


Fig. 23. Calibration curves for the colorimetric determination of L-leucine with Nitro-TB and MTT as tetrazolium salt. The determination was performed as described in EXPERIMENTAL PROCEDURES except for using 0.4  $\mu\text{mol}$  of Nitro-TB (●-●) and MTT (o-o) instead of INT.



*Determination of Branched-Chain Keto Acids* The branched-chain keto acids were determined with the enzyme by measuring a decrease of the absorbance at 340 nm owing to decrease of NADH. The amount of enzyme used was smaller than that for the determination of branched-chain amino acid, because the reaction equilibrium lies so far to the formation of the amino acid from the keto acid, and the rate is higher than that of the reverse reaction. Fig. 24 shows a linear relationship between a decrease of the absorbance at 340 nm and the amount of  $\alpha$ -ketoisocaproate (0.01-0.12  $\mu$ mol).  $\alpha$ -Ketoisovalerate,  $\alpha$ -ketovalerate and  $\alpha$ -ketobutyrate also could be determined in the same way. Several amino acids and keto acids did not effect for the determination of  $\alpha$ -ketoisocaproate (Table XII).

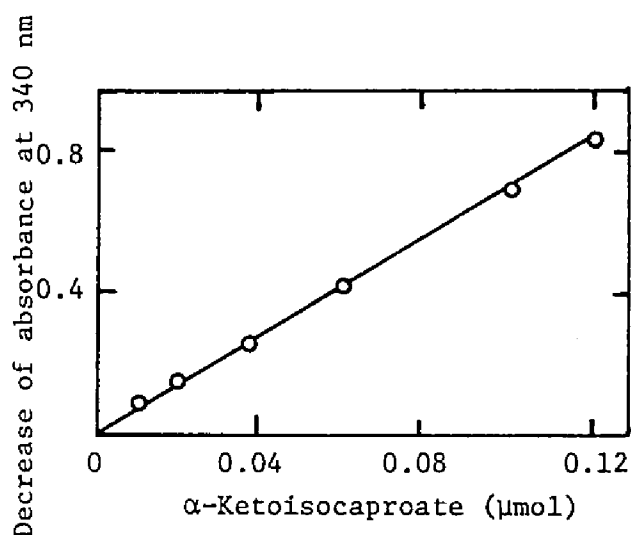


Fig. 24. The relationship between  $\alpha$ -ketoisocaproate concentration and a decrease of the absorbance at 340 nm.

Table XII. *Effect of some amino acids and  $\alpha$ -keto acids on the determination of  $\alpha$ -ketoisocaproate.* The reaction mixture (1 ml) consisted of NADH (0.2  $\mu$ mol),  $\alpha$ -ketoisocaproate (0.1  $\mu$ mol), leucine dehydrogenase (4  $\mu$ g),  $\text{NH}_4\text{OH-NH}_4\text{Cl}$  buffer (pH 9.5, 650  $\mu$ mol) and amino acid or keto acid (0.1 ml). Other conditions were as described in EXPERIMENTAL PROCEDURES.

Amino acids or keto acids added	Amount ( $\mu$ mol)	Relative NADH reduction
No addition	-	100
L-Leucine	0.1	103
	0.5	101
	1.0	97
D-Leucine	0.1	98
	1.0	99
L-Valine	1.0	99
D-Valine	1.0	100
L-Alanine	1.0	98
L-Glutamate	1.0	100
$\alpha$ -Ketoglutarate	1.0	99
Oxalacetate	1.0	103
Pyruvate	1.0	105

#### SUMMARY

The simple and sensitive methods for the determination of L-branched-chain amino acids and their keto analogues with the enzyme were presented. L-Branched-chain amino acids can be determined by measuring the formation of NADH spectrophotometrically at 340 nm. L-Branched-chain amino acids also are determined by colorimetric method in which phenazine metho-

sulfate and iodonitrotetrazolium chloride are used as primary electron acceptor and a terminal oxidant, respectively. The branched-chain keto acids are determined by measuring a decrease of NADH spectrophotometrically at 340 nm.

## CONCLUSION

The distribution of bacterial leucine dehydrogenase was investigated. The enzyme occurs mainly in *Bacillus* species and was purified and crystallized from *B. sphaericus* (IFO 3525), which has the highest enzyme activity. The crystalline preparation was found homogeneous by the criteria of ultracentrifugation ( $S_{20}^w = 9.0$  S) and polyacrylamide disc gel electrophoresis.

Leucine dehydrogenase exhibits the optimum pH in alkaline side for both the forward and the reverse reactions. The enzyme catalyzes the oxidative deamination of L-leucine, L-valine, L-isoleucine, L-norvaline, L- $\alpha$ -aminobutyrate and L-norleucine, and the reductive amination of their keto analogues. The enzyme requires  $NAD^+$  as a cofactor, which cannot be replaced by  $NADP^+$ . D-Enantiomers of the substrate amino acids inhibit competitively the oxidation of L-leucine. The enzyme activity is significantly reduced by both sulfhydryl reagents and pyridoxal 5'-phosphate. Purine and pyrimidine bases, nucleosides and nucleotides have no effect on the enzyme activity.

The molecular weight of the enzyme was determined to be about 245,000 by sedimentation equilibrium method. The enzyme consists of 6 identical subunits ( $M_r$ ; 41,000) and amino- and carboxyl-terminal amino acid residues are methionine and glutamate, respectively. The enzyme contains 6 sulfhydryl groups required for catalysis and 6 coenzyme binding sites per enzyme molecule. These results suggest the presence of one active site per the subunit.

Initial velocity and product inhibition studies show that the reductive amination proceeds through a sequential ordered ternary-binary mechanism, in which NADH binds first to the enzyme followed by  $\alpha$ -ketoisocaproate and ammonia, and the products are

released in order of L-leucine and  $\text{NAD}^+$ . The Michaelis constants are as follows: L-leucine (1 mM),  $\text{NAD}^+$  (0.39 mM), NADH (35  $\mu\text{M}$ ),  $\alpha$ -ketoisocaproate (0.31 mM) and ammonia (0.2 M). Stereospecificity of hydrogen transfer between coenzyme and substrate catalyzed by leucine dehydrogenase was investigated. The *pro-S* hydrogen at C-4 of the dihydronicotinamide ring of NADH is exclusively transferred to the substrate; the enzyme is B-stereospecific.

The enzyme shows the antitumor activity against Ehrlich ascites carcinoma in DD mice.

The simple and sensitive methods for the determination of L-branched-chain amino acids and their keto analogues with the enzyme were investigated. L-Branched-chain amino acids can be determined by measuring the formation of NADH spectrophotometrically at 340 nm. L-Branched-chain amino acids are determined also by the colorimetric method with the system containing phenazine methosulfate as primary electron acceptor and iodonitrotetrazolium chloride as terminal oxidant. Branched-chain keto acids can be determined by measuring a decrease of NADH spectrophotometrically at 340 nm.

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